AFFINITY PRECIPITATION USING THERMO-RESPONSIVE, WATER-SOLUBLE POLYMERS AS MATRIX; SYNTHETIC STUDIES TOWARD THE AGELIFERINS

A Thesis

by

MIN ZHOU

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2003

Major Subject: Chemistry

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Approved as to style and content by:

Daniel Romo (Chair of Committee)	
David E. Bergbreiter (Member)	James C. Hu (Member)
Pam Matthews (Member)	Emile A. Schweikert (Head of Department)

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ABSTRACT

Affinity Precipitation Using Thermo-Responsive, Water-Soluble Polymers as Matrix;

Synthetic Studies Toward the Ageliferins

(December 2003)

Min Zhou, B.S., Beijing Medical University, P.R. China

Chair of Advisory Committee: Dr. Daniel Romo

Isolated from marine sponges, many bromopyrrole alkaloids have become natural products of intense scientific interest. The oroidin-derived class of dimeric bromopyrrole alkaloids that include ageliferin, bromoageliferin, and dibromoageliferin exhibit interesting biological properties, including actomycin ATPase, antiviral and antibacterial activities.

As a prelude to the total synthesis of ageliferin, an intramolecular Diels-Alder (IMDA) reaction was explored as a means to introduce the three contiguous stereocenters. Toward this end, various IMDA precursors were synthesized, and several strategies for the synthesis of IMDA substrates were devised and explored.

Affinity chromatography is a powerful technique that enables the purification of a specific protein from a complex mixture. However, traditional affinity chromatography techniques are somewhat limited due to the solid state of the matrix. To overcome these limitations, the utility of a soluble polymer as an alternative affinity matrix was explored for the isolation of natural product receptors. This polymer displays physical properties that make it an ideal matrix for protein isolation and

purification. The parent polymer is soluble in aqueous solution at 4 °C and precipitates once the mixture reaches 32 °C. Furthermore, employing this polymer for affinity chromatography may limit the nonspecific binding of proteins. It is also possible to determine the ligand loading using standard analytical techniques such as ^{1}H and ^{13}C NMR. For proof of concept, a dexamethasone-containing macroligand was synthesized to isolate the known glucocorticoid receptor. In addition, a cyclosporin A-containing macroligand was synthesized and employed to isolate the well known and more robust cyclophilins. In this work, it was demonstrated that indeed the ligand loading can be determined by ^{1}H NMR technique. In addition, it was established, as expected, that the water solubility of the macroligands varies based on the hydrophilicity and hydrophobicity of the ligand and degree of ligand loading.

DEDICATION

To my parents and my friends

ACKNOWLEDGEMENTS

First, I would like to thank my advisor, Dr. Romo, for his guidance and support during my graduate study. I would also like to thank my committee members, Dr. Bergbreiter, Dr. Matthews and Dr. Hu for their helpful suggestions and valuble instruction.

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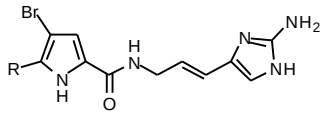
AN INTRODUCTION TO THE OROIDIN DERIVED BROMOPYRROLE ALKALOIDS : AGELIFERIN, BROMOAGELIFERIN, DIBROMOAGELIFERIN, SCEPTRIN AND DIBROMOSCEPTRIN

1. Introduction

Traditionally, soils, plants and micro-organisms have been fertile sources for the isolation and identification of many biological active molecules. However, during the last few decades, the oceans have also become recognized as an abundant source of bioactive natural products. A vast array of oceanic organisms including sponges, algae, microorganisms, mollusks have provided structurally novel and diverse natural products that display equally interesting bioactivity.[1] From all the marine organisms mentioned above, sponges have been the most thoroughly investigated species.[2] Biosynthetically related to oroidin (**1**) and hymenidin (**2**), the family of bromopyrrole alkaloids found in Caribbean sponges *Agelas conifera* and *Agelas n. sp*. have aroused scientific interest as a result of their novel chemical architecture and potential therapeutic value (Figure 1).[3,4]

This thesis follows the style and format of the *Journal of the American Chemical Society*.



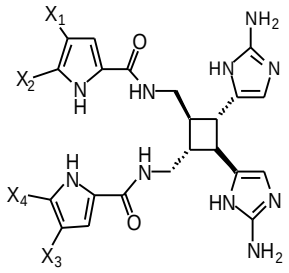
1: R=Br, oroidin
2: R=H, hymenidin

Figure 1. Structures of oroidin and hymenidin

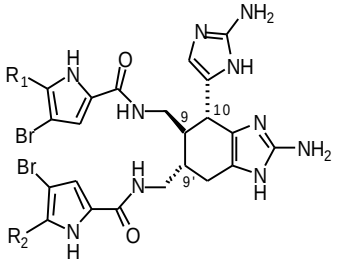
Oroidin was first isolated by Minale from *Agelas oroides*.[5] During the following ten years, several scientists including Minale, Garcia and Faulkner tried to assign the structure of oroidin.[5,6,7] Finally, Faulkner and co-workers employed x-ray crystallography to elucidate the structure of oroidin (**1**).[7]

2. Isolation and Bioactivity

The oroidin alkaloid family of marine natural products include the ageliferins and sceptrins. These marine metabolites display varying degrees of chemical structures and bromination, highlighting the chemical diversity of natural products isolated from the ocean (Figure 2).[8]



3: X_1 = Br, X_2 = H, X_3 = Br, X_4 = H, sceptrin
4: X_1 = X_2 = X_3 = Br, X_4 = H, bromosceptrin
5: X_1 = X_2 = X_3 = X_4 = Br, dibromosceptrin



6: R_1 = R_2 = H, ageliferin
7: R_1 = Br, R_2 = H, bromoageliferin
8: R_1 = R_2 = Br, dibromoageliferin

Figure 2. Structures of sceptrins and ageliferins

Harvested from the sponges *Agelas coniferin* and *A. cf. Mauritiana*, ageliferin **6**, bromoageliferin **7** and dibromoageliferin **8** were first reported by Rinehart and co-workers in 1989.[9] Subsequently, Kobayashi isolated ageliferins from an Okinawan sponge *Agelas sp.*, established the structures of compounds **6**-**8**, and studied their biological activity.[8] The marine sponge *Agelas sp.* was collected in Kerama Islands, Okinawa. A total of 700 grams of sponge were first extracted with methanol and then partitioned between ethyl acetate and water. The ethyl acetate-soluble fractions were subjected to a series of chromatographic purifications, ODS column to furnish ageliferin **6** (0.02% wet weight), bromoageliferin **7 (**0.01%), and dibromoageliferin **8 (**0.01%). The relative structure of the ageliferins was established from detailed analysis of the ^{1}H and ^{13}C NMR data, IR, UV, and high-resolution FABMS analysis comparison with the spectra of sceptrin **3**, oroidin **1** and hymenidin **2**. All ^{1}H and ^{13}C NMR signals of ageliferin were assigned from two-dimensional ^{1}H-^{1}H DQF-COSY, HMQC, HMBC spectra, and one-dimentional double and triple resonance experiments. The C-9, C-9'

and C-10 substituents of the central cyclohexene ring of ageliferin were all determined to occupy equatorial positions based on analysis of the ^{1}H-^{1}H coupling constants, characteristic of trans-diaxial protons ($J_{9,10}$ = 7 Hz, $J_{9,9'}$ = 8 Hz and $J_{9',10'a}$ = 8 Hz) (Figure 3).[8]

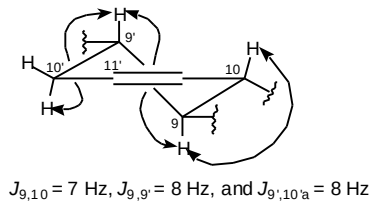


Figure 3. ^{1}H-^{1}H coupling constants (J/Hz) for the cyclohexene ring of ageliferin

The actin-myosin protein complex is involved in muscle contraction and other cell-motility events. The energy source for these cell motility events comes from myosin ATPase. Experiments have shown that the ageliferins are potent actomyosin ATPase activators. Furthermore, it was demonstrated that the ATPase activity of rabbit skeletal muscle myofibrils was elevated to 150, 190 and 200% of the control value by ageliferin (30 μM), bromoageliferin (1 μM) and dibromoageliferin (1 μM) respectively.[8] According to this report, the ageliferins may be useful as chemical tools for biological studies of the molecular mechanism of actin-myosin contractile systems.

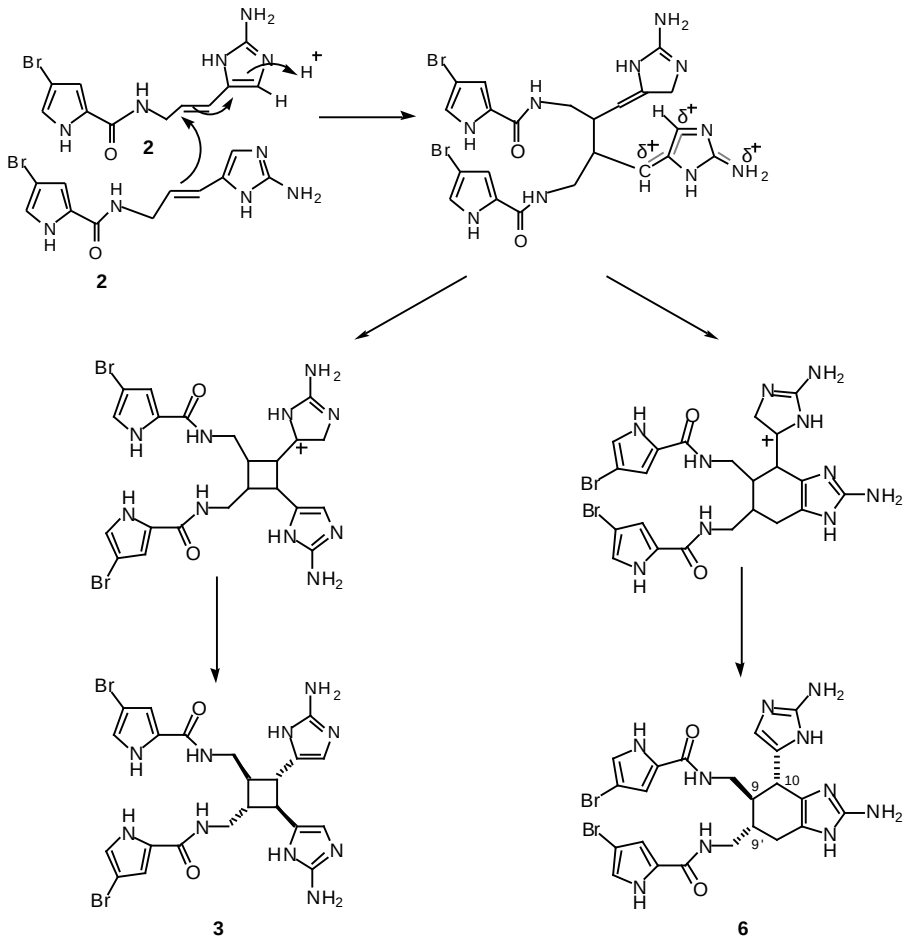
Faulkner and Clardy reported the isolation, structure and bioactivity of sceptrin (**3**), isolated from the sponge *Agelas sceptrum* collected at Glover Reef, Belize (Figure 2).[10] The lyophilized sponge was extracted with hexane, dichloromethane, and methanol

and the acetone-insoluble portion of the methanolic extracts were submitted to chromatography on Sephadex LH-20 twice followed by a LiChrosorb DIOL column to obtain oroidin (0.5% dry weight) and sceptrin (2.1% dry weight).[10] Antimicrobial assays of sceptrin revealed a broad range of antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Candida albicans*, *Pseudomonas aeruginosa*, *Alternaria sp*. (fungus), and *Cladosporium cucumerinum*.[10]

3. Biosynthesis

Several proposals for the biosynthesis of ageliferins **6**-**8** and sceptrins **3**-**5** have been suggested. Sceptrin **3** is structurally derived from hymenidin **2** by way of a head to head [2+2] cycloaddition of **2** (Scheme 1).[3] Along the same lines, the gross structure of ageliferin is considered to arise from [4+2] cycloaddition of two molecules of hymenidin (**2**) followed by double-bond isomerization.[3,8] However, Faulkner and Clardy suggested that a simple photodimerization of hymenidin in the biosynthetic pathway was unlikely due to insufficient light at the ocean depths where *Agelas sceptrum* was harvested and the optical activity of sceptrin is suggestive of enzyme involvement in the biosynthesis from achiral hymenidin.[10]

Scheme 1

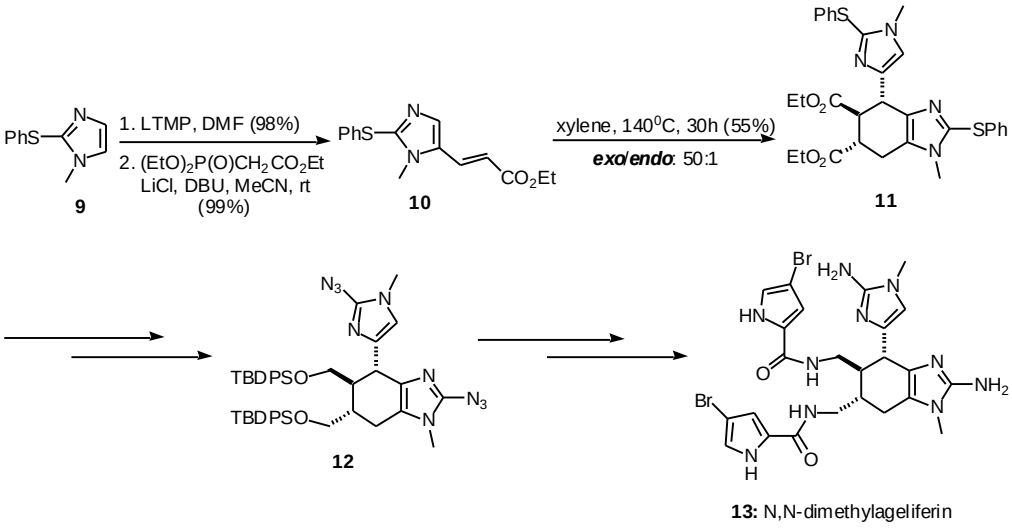


4. Previous Synthetic Studies

Publications detailing synthetic approaches toward the ageliferins have so far been limited to two reports with a total synthesis remaining elusive.[11a,11b] Ohta reported an elegant biomimetic synthesis of racemic 12,12'-dimethylageliferin (**13**) via an intermolecular Diels-Alder (DA) dimerization of vinyl-imidazoles (Scheme 2).[11a] The Diels-Alder substrate alkenyl imidazole **10** was obtained from capture of the lithio anion

by DMF followed by Horner-Wadsworth-Emmons olefination of the aldehyde. Heating vinyl imidazole **10** in xylene at 140 °C for 30 hours produced **11** in moderate yield with high levels of regio- and facial selectivity (*exo*/*endo* = 50:1). During the course of the Diels-Alder cycloaddition, double bond isomerization occurs driven by regeneration of aromaticity of the imidazole ring. Functional group manipulation gave bis-azidoimidazole **12**. This intermediate was elaborated to unnatural, racemic 12, 12'-dimethylageliferin **13** in five steps.

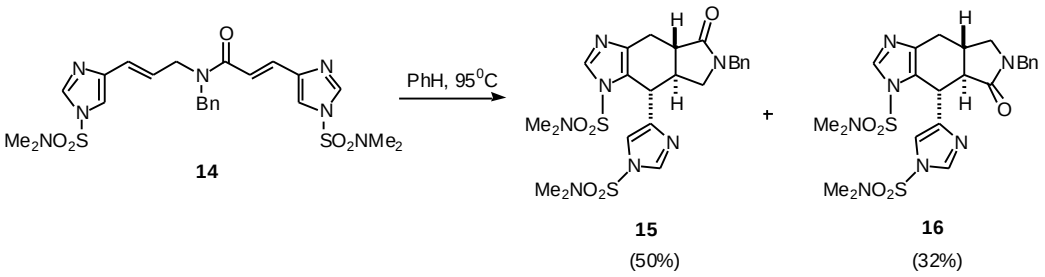
Scheme 2



More recently, Lovely has investigated the intramolecular Diels-Alder reactions of unsaturated amides derived from urocanic acid to provide trans ring-fused products as the major adduct via an endo transition state (Scheme 3).[11b] Interestingly, cycloadducts

15 and **16** contain the proper structural and stereochemical elements for subsequent conversion to ageliferin.

Scheme 3



5. Conclusion

The family of bromopyrrole-imidazole alkaloids found in marine sponges have challenged the synthetic community due to their intriguing structural complexity and diversity as well as their remarkably wide range of biological activity. Several proposals for their biosynthetic origins including intermolecular [2+2] and [4+2] cycloaddition have been suggested. To date, two approaches have enabled the synthesis of unnatural (+/-)-12,12'-N,N'-dimethylageliferin and potential ageliferin precursor via inter- and intramolecular Diels-Alder processes.

CHAPTER II

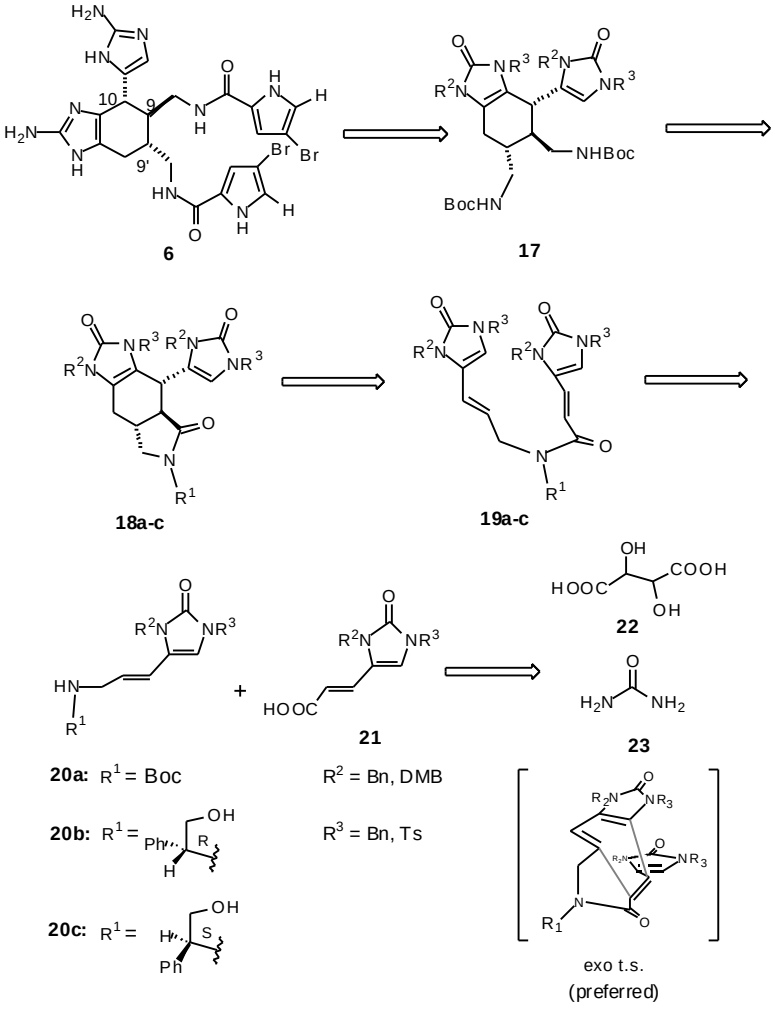
SYNTHETIC INVESTIGATIONS OF AGELIFERIN

1. Retrosynthesis Analysis of Ageliferin

The strategy for a synthesis of ageliferin is centered on the intramolecular Diels-Alder (IMDA) cycloaddition of vinylogous amide **19a** (Scheme 4). Advancements in a symmetric Diels-Alder cycloadditions would permit use of catalytic amounts of a Lewis acid to facilitate the construction of the central cyclohexene ring.[12a,12b,12c] Amide **19a** could be obtained from the coupling of amine **20a** and acid **21**, in turn derived from racemic tartaric acid (**22**) and urea (**23**). Ring-opening aminolysis of lactam **18** would be followed by conversion of the imidazolones to aminoimidazoles and incorporation of the bromopyrroles to deliver racemic ageliferin. Having established the validity of this approach, R-(-)- phenylglycinol or S-(+)-phenylglycinol might be used to induce asymmetry in the IMDA yielding optically active **18a**. A proposed transition state arrangement is shown (Scheme 4). It is reasonable to suggest that either enantiomer of ageliferin will be produced by proper choice of chiral inductor (Scheme 4). Other members of the Romo group have investigated synthetic strategies toward axinellamine, palau'amine, and phakellin. Anja Dilley demonstrated that protection of the imidazolone nitrogen as a sulfonamide adds more stability to the diene system thus increasing the yield of the IMDA cycloaddition and most importantly the regioselectivity.[45] In addition, deprotection of the benzyl groups from the nitrogen on the imidazolone rings usually required relatively harsh conditions. It was also

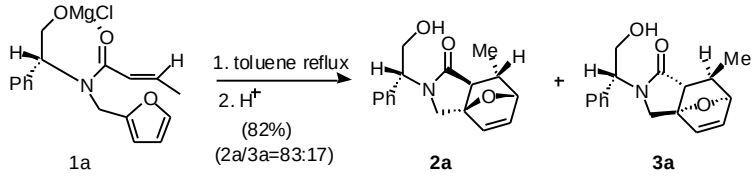
demonstrated that tosyl and dimethoxylbenzyl (DMB) protecting groups are ideal protecting groups likely compatible with functionality present in the latter stages of the synthesis of axinellamine and palau'amine.

Scheme 4



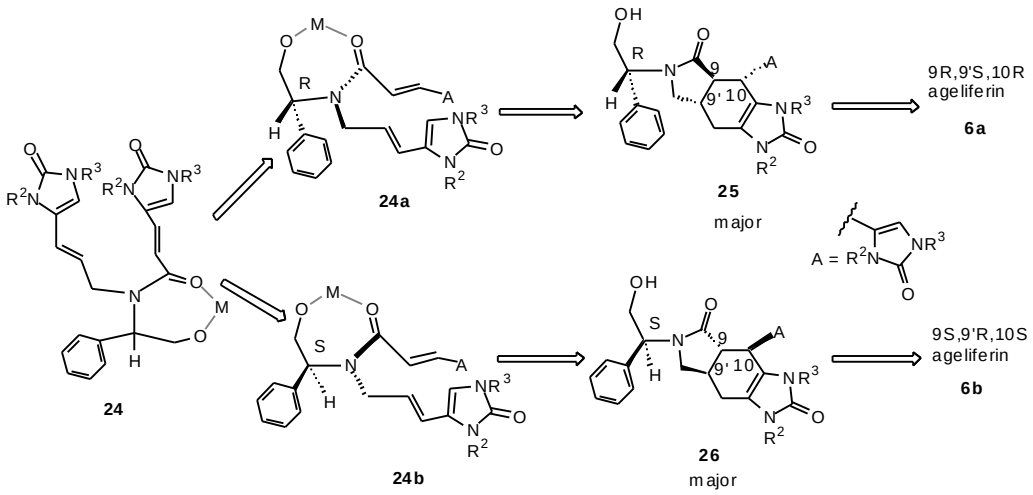
In 1981, Mukaiyama and Iwasawa reported the asymmetric IMDA process using (-)-phenylglycinol as linker, which led both to the effective transfer of chirality and to acceleration of the reaction (Scheme 5).[12]

Scheme 5



We considered extentending this method to an enantioselective synthesis of ageliferin. Following addition of a base and various metals to **19b-c**, a 7-membered complex is proposed (Scheme 5). The chirality transfer process is proposed to take place via transition state arrangements **24a** and **24b** dictated by steric repulsions between the benzene ring of phenylglycinol and the methylene group next to the double bond in chelate complex **24a**. In order to minimize non-bonding steric repulsions, the approach of the imidazolone ring to the dienophile should take place preferentially from the side opposite to the benzene ring. As a result of this facial selectivity, cycloadduct **25** is expected to be the major product, when (R)-phenyl glycinol is employed. Conversely, (S)-phenyl glycinol should provide enantiomeric cycloadduct **26** (Scheme 6).

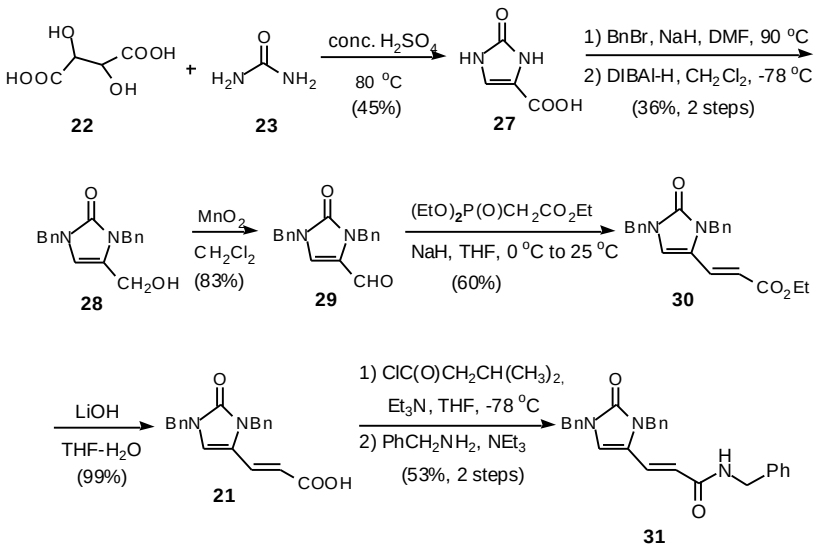
Scheme 6



2. Synthesis of Vinyl Imidazolone Substrates

The condensation between racemic tartaric acid (**22**) and urea (**23**) was used to prepare vinyl imidazolones **21** in multigram scale. Carboxylic acid **27** was then subjected to perbenzylation followed by diisobutylaluminum hydride (DIBAl-H) reduction to yield alcohol **28** (Scheme 7).[13] Allylic alcohol **28** was oxidized to aldehyde **29** with manganese(IV) oxide, and the chain was elongated to ester **30** by way of a Horner-Wadsworth-Emmons (HWE) olefination. Activation of acid **21** by formation of a mixed anhydride and coupling with benzylamine delivered amide **31**.

Scheme 7

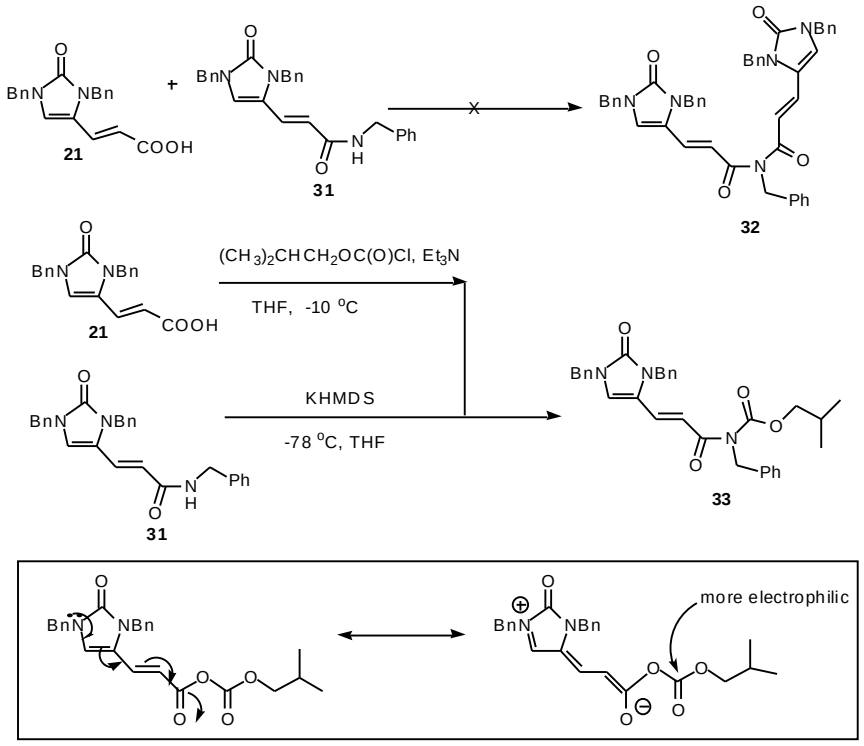


3. Exploring the Synthesis of Diels-Alder Reaction Substrates

Our attempts to synthesize the symmetric imide **32** from coupling of amide **31** and acid **21** proved more difficult than anticipated. Unfortunately, amide **31** was unstable to bases such as *n*-BuLi and NaH. Although amide **31** was stable when treated with KHMDS at low temperature, upon addition of the mixed anhydride derived from acid **21** and isobutyl chloride, isobutyl carbamate **33** was isolated as the only product (Scheme 8). This could be understood by delocalization of the lone pair of electrons on the terminal nitrogen and the α,β-unsaturation, which in turn deactivates the desired carbonyl toward nucleophilic attack. Thus, the *N*-potassium amide nucleophile preferentially attacks the carbamate carbonyl on the mixed anhydride molecule possibly due to both electronic and steric effects. In addition, the desired imide **32** was not

produced even after increasing the steric hindrance of the neighbouring carbonyl employing the mixed anhydride derived from pivaloyl chloride.

Scheme 8



4. Conclusion

The instability of α,β-unsaturated amine and alcohol caused difficulties for the synthesis of IMDA substrate. Anja Dilley's work has shown that replacing one of the benzyl protecting groups with an electron withdrawing tosyl group brings more stability to the diene system and increases the regioselectivity of the intermolecular Diels-Alder reaction. In addition, the final deprotection of the benzyl groups from the nitrogen on

the imidazolone rings usually needed relatively harsh conditions. These findings should be incorporated into future studies towards the ageliferins.

CHAPTER III

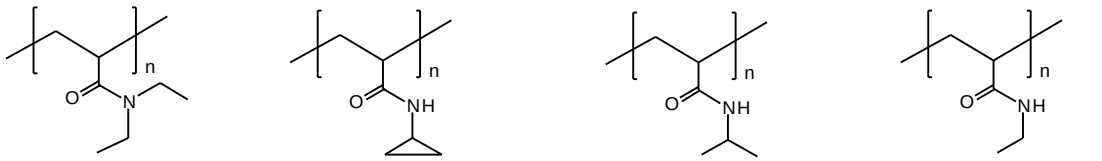
AN INTRODUCTION TO THERMO-RESPONSIVE, WATER-SOLUBLE POLY-*N*-ISOPROPYLACRYLAMIDE (PNIPAM) POLYMERS FOR PROTEIN PURIFICATION

1. Thermo-responsive, Water-soluble Polymers

Nature uses polymers for structure and also cellular machinery. Most functional biopolymers respond to external stimuli in an all-or-nothing, or at least highly non-linear mode.[14] The reason for these nonlinear responses comes from highly cooperative interactions existing at the molecular level. A weak interaction between two separate but specific monomer units can turn into a large driving force for a process occurring in the whole system when thousands of these monomer units act in concert.[14] Recently, there has been significant progress made toward extending the properties of these biopolymers to fully synthetic functional polymers. These synthetic polymers respond in a desired way to external environmental stimuli such as temperature, pH, and electric or magnetic fields. These polymers undergo fast and reversible changes from a hydrophilic to a hydrophobic state.[15] These highly nonlinear responses of synthetic polymers occur mainly in water, sometimes in organic solvents[16] or polymer blends[17]. When this stimulus-responsive behavior occurs in aqueous solution, these polymers have potential as interesting tools for biotechnological and medicinal research.[14] Among the synthetic stimulus-responsive polymers, temperature responsive polymers have been the most extensively studied. Each of these polymers has their characteristic lower

critical solution temperature (LCST). The polymer is soluble below its LCST due to a combination of entropic and enthalpic effects. Above the LCST, the polymer will precipitate out of solution presumably due to magnification of hydrophobic interactions between the side chains at higher temperatures.[18,19, 20] Addition of sodium chloride can linearly decrease the LCST of polymers such as poly (*N*, *N*-diethylacrylamide) (PDEA). However, the addition of SDS at concentrations up to its critical micelle concentration can gradually increase the LCST of the PDEA.[18]

In aqueous solution, several *N*-substituted poly (acrylamides) undergo a thermally induced, reversible phase transition. The LCSTs of homopolymers such as poly (*N*, *N*-diethylacrylamide) **34**, poly (*N*-cyclopropylacrylamide) **35**, poly (*N*-isopropylacrylamide) **36**, and poly (*N*-ethylacrylamide) **37** in distilled H_2O have been reported to be 33, 58, 32, and 74 °C, respectively (Figure 4).[18] Among these homopolymers, poly (*N*-isopropylacrylamide) (PNIPAM) **34** has received special attention due to its sharp phase transition temperature at 32 °C.[19]

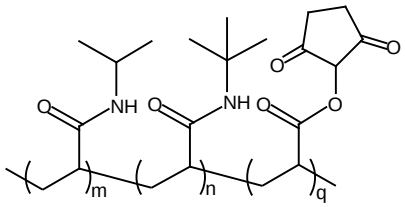


34: poly (*N, N*-diethylacrylamide) (33 °C)
35: poly (*N*-cyclopropylacrylamide) (58 °C)
36: poly (*N*-isopropylacrylamide) (32 °C)
37: poly (*N*-ethylacrylamide) (74 °C)

Figure 4. Structures of homopolymers

The copolymers of PNIPAM and functionalized monomers have been found to not only be thermoresponsive but also show inverse temperature-dependent solubility in water.[19,21] The LCST of these polymers can be adjusted by changing the structure and loading of the functionalized monomer (Table 1).[24] these thermo-responsive polymers have enabled the design of smart chemical catalysts with their catalytic activities switched off and on by precipitation and redissolution depending on reaction temperature,[22,23] In addition, these polymers have been used as tools for protein purification[25] and synthesis.[24]

Table 1. Structure and LCST of some PNIPAM polymer variants



38a: m=10, n=0, q=1
38b: m=20, n=0, q=1
38c: m=20, n=1, q=1
38d: m=20, n=5, q=2

Entry	Polymer	m	n	q	LCST
1	**38a**	10	0	1	30 °C
2	**38b**	20	0	1	28 °C
3	**38c**	20	1	1	22 °C
4	**38d**	20	5	2	20 °C

2. Affinity Chromatography

Bioactive natural products are useful probes for various cellular processes, by serving as affinity reagents for purification of cellular receptors. These studies can lead to unraveling of various cellular process. Affinity chromatography, a simple, relatively inexpensive and very rapid process, has been the method of choice for isolating and purifying many biomolecules from complex mixtures.[26a,26b,26c] Because of the solid state of the matrix typically employed, traditional affinity chromatography techniques suffer from a few limitations. The first problem is nonspecific binding, since swollen resins and porous affinity beads can easily trap nonspecific proteins. The second limitation arises from the insolubility of the matrix which in turn does not allow analysis and integrity of the loaded ligand or allow measurement of ligand loading. One possible solution to these limitations is the use of thermoresponsive, water-soluble copolymers e.g. **38a** (Table 1). The physical properties of these polymers argue for their application as a matrix in protein isolation and purification. Polymer **38a** is soluble in aqueous solution at 4 $^{\circ}$C and precipitates once the mixture reaches 32 $^{\circ}$C. By employing this polymer, it may prove possible to decrease the nonspecific binding relative to traditional techniques. Furthermore, due to their solubility, thus may enable determination of ligand loading using such standard analytical techniques as ^{1}H NMR. Mattiasson and coworkers have examined copolymers **40** of PNIPAM/1-vinylimidazole copolymers loaded with Cu (II) ion as a potential matrix for affinity purification to purify metal affinity proteins from a variety of cereals (Figure 5).[25] The success of this first report suggested the potential of thermoresponsive polymers as an affinity matrix. While elegant, this experiment was still plagued by some limitations. First, the target proteins needed to have a high affinity for the metal loaded on the polymer. Secondly, the metal ions add a net positive charge to the copolymer, which increases the LCST. Reasoning

that an increase in the ionic strength of the solution would facilitate the precipitation of these metal-bound copolymers, Mattiason and coworkers tried to compensate for this LCST change by adding NaCl. As a result, these two limitations prevent copolymer **40** from becoming an effective general matrix for protein isolation. Subsequently, the same research group reported thermo-responsive copolymer **41** consisting of *N*-vinylcaprolactam and 1-vinylimidazole (VI) and fully characterized **41** using static and dynamic light scattering and ^{1}H NMR (Figure 5).[27] This research provides the ground work for the application of thermo-responsive, water-soluble polymer for metal-chelate affinity precipitation techniques. Unfortunately, the LCST of polymer **41** is 48 ˚C, a temperature which would lead to denaturization of most proteins. In 2001, Freitag reported synthesis of affinity macroligand bearing 2-iminobiotin at the termini to purify avidin from crude cell extracts (Figure 5).[28] The advantage to this approach due to its low ligand loading is that addition of ligands changes the properties of the polymer only incrementally.

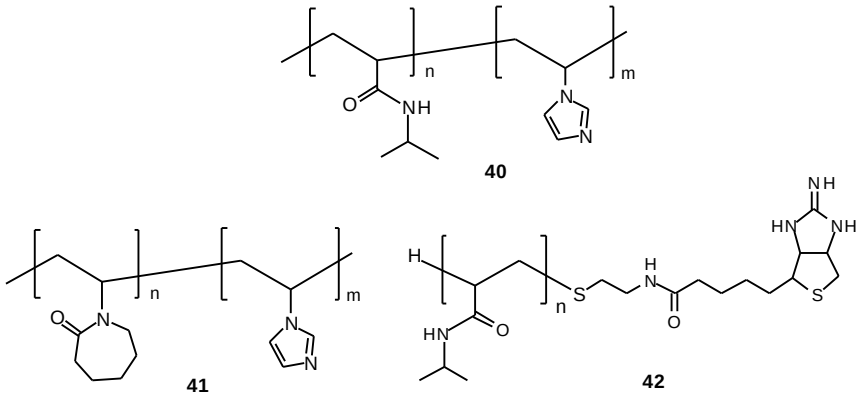


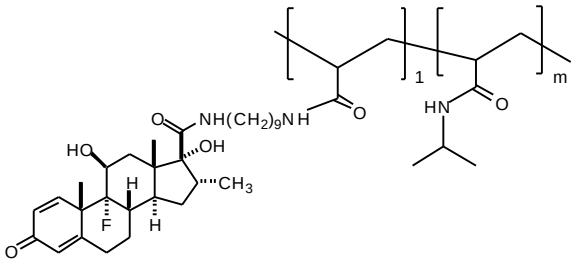
Figure 5. Structures of copolymers and macroligands

CHAPTER IV

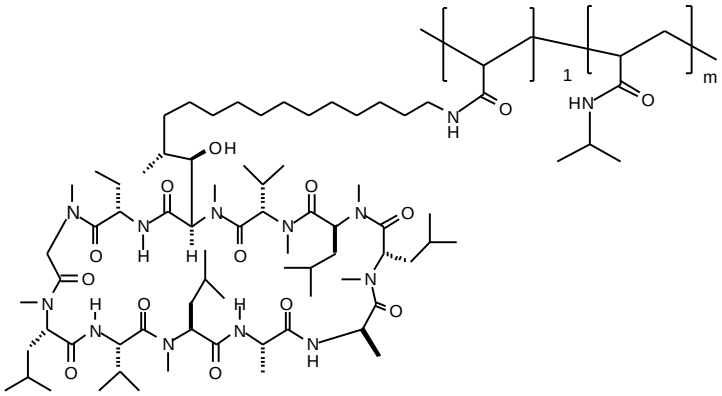
INVESTIGATIONS OF PNIPAM POLYMERS AS MATRICES FOR NATURAL PRODUCT CELLULAR RECEPTOR ISOLATION

1. Introduction

The goal of this research project was to explore the possibility of using thermoresponsive polymers for isolation of small molecule (e.g. natural products) protein receptors. As proof of principle, a dexamethasone macroligand **43** was designed to isolate the glucocorticoid receptor. In addition, we designed a cyclosporin A macroligand for isolation of the cyclophilins. The purpose of choosing these two ligands follows: (1) The glucocorticoid receptor is very sensitive to temperature and protease activity,[30] therefore the dexamesosone macroligand can be used to test whether this system can be used to isolate sensitive natural product receptors; (2) Cyclosporin A has a number of robust protein receptors, known as cyclophilins, with different levels of abundancy in many commercial available cell lines.[29] Thus, it is an ideal model to test both the feasibility and sensitivity of the soluble polymer system. Dexamethasone macroligand **43**, and cyclosporine A macroligand **44** are shown in Figure 6.



43: Dexamethasone Macroligand



44: Cyclosporin A Macroligand

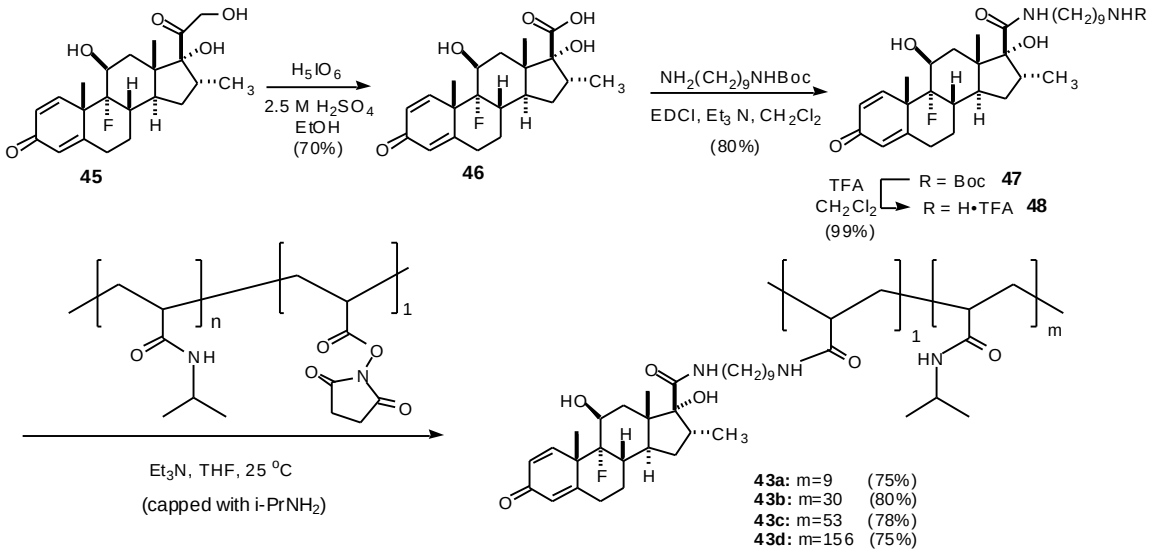
Figure 6. Structure of dexamethasone macroligand (**43**) and cyclosporin A macroligand (**44**)

2. Synthesis and Characterization of a Dexamethasone-PNIPAM Macroligand: Application to the Isolation of the Glucocorticoid Receptor

The glucocorticoid receptors are the best characterized eukaryotic transcription factors.[30] The binding of steroids to these receptors leads to activation of these complexes in the cytosol,[31] enabling entry into the nucleus and binding to DNA via a zinc finger domain to effect gene transcription.[32, 33] The high affinity between glucocorticoids and their receptors make them ideal for proof of principle of new matrices to be used for protein affinity isolation.[31] Synthesis of the dexamethasone

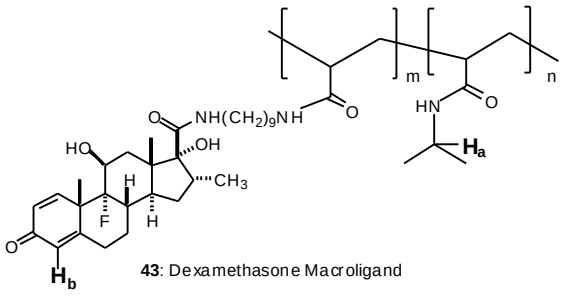
macroligand began with the oxidative cleavage of the hydroxyketone of dexamethasone **45** to give the dexamethasone acid **46** in moderate yield (Scheme 8).[34] Amide **47** was formed by EDCI coupling between mono-*N*-Boc-1,9-diamine and acid **46**. Removal of the *N*-Boc carbamate was achieved upon exposure to trifluroacetic acid (TFA), which delivered the amine-TFA salt **37**.[35] Initially, several methods were explored to freebase the amine-TFA salt to facilitate coupling to the polymer. However, the amine-TFA salt **48** was determined to be unstable during prolonged exposure to basic conditions. Although free-basing with aqueous sodium bicarbonate solution followed by rapid EtOAc extraction could partially freebase before decomposition, the amine-TFA salt **48** was best directly employed in coupling to polymer under basic conditions. The coupling reaction proceeded smoothly affording the dexamethasone macroligand **43a-d** with varying ligand loadings (Scheme 8). Although repetitive precipitation between a two organic solvent system (THF and hexane) followed by filtration is one typical method applied for purification of the parent polymer,[17] we found that significant quantities of macroligand were lost during the repetitive filtration process in small scale reactions. Later we found that except for the final filtration, centrifugation can be applied to separate the macroligand from the solvent. In this way, the yield of this coupling reaction was markedly improved.

Scheme 8



Characterization including integrity of dexamethasone and degree of loading of dexamethasone was determined by ^{1}H NMR (Figure 7). In addition, we were able to measure the solubility of the macroligand in water at 4 °C. As expected, when the loading was relatively low (i.e. **43b**, **43c**, and **43d**), the dexamethasone-polymer macroligand exhibited physical properties similar to the parent polymer **38c**. When the loading was high (i.e. **43a**), the macroligand was insoluble in most organic solvents and water. This may be explained by the fact that this macroligand is highly hydrophobic. Thus, with relatively high loading of the ligand, the ligands themselves may tend to aggregate together. Obviously, when employing hydrophobic natural products, one should minimize ligand loading to ensure that the matrix solubility remains similar to that of the parent polymer **38c**.

(a)



43: Dexamethasone Macroligand

Compound	m:n[a]	H_2O solubility at 4 °C (mg/mL)
43a	1:10 (1:9)	insoluble
43b	1:25 (1:30)	25
43c	1:34 (1:53)	27
43d	1:133 (1:156)	42

[a] Expected m:n ratio based on stoichiometry of reagents employed. Actual ratio based on ^{1}H NMR integration given in parentheses.

(b)

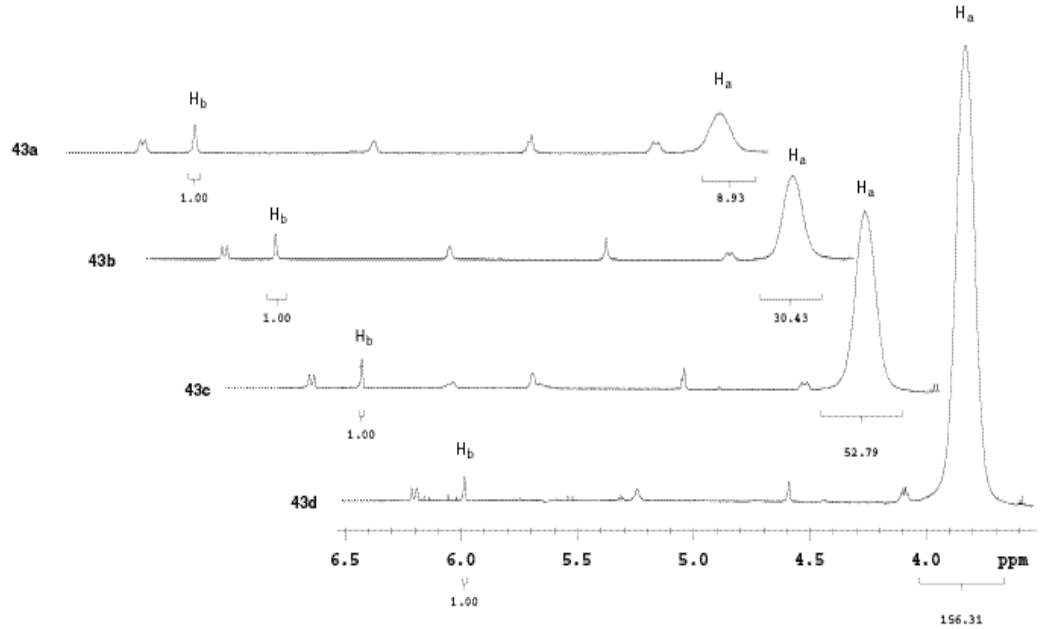
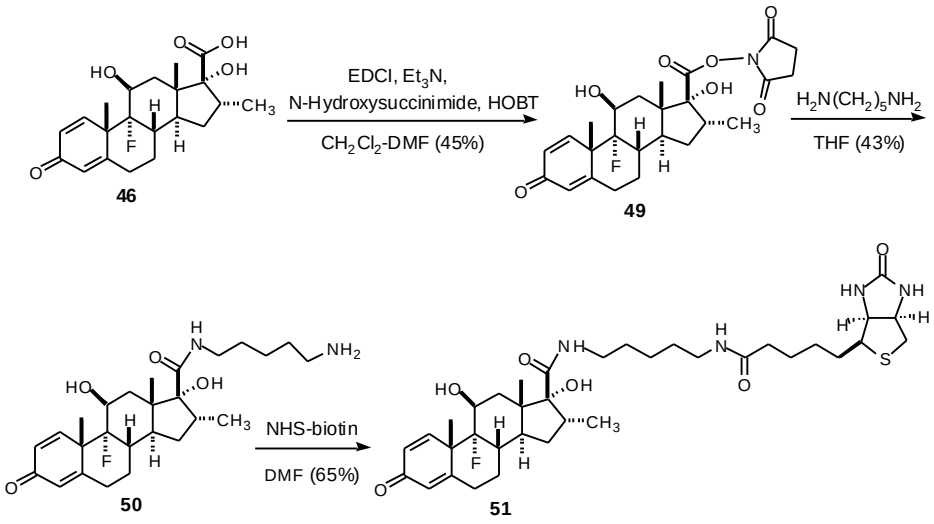


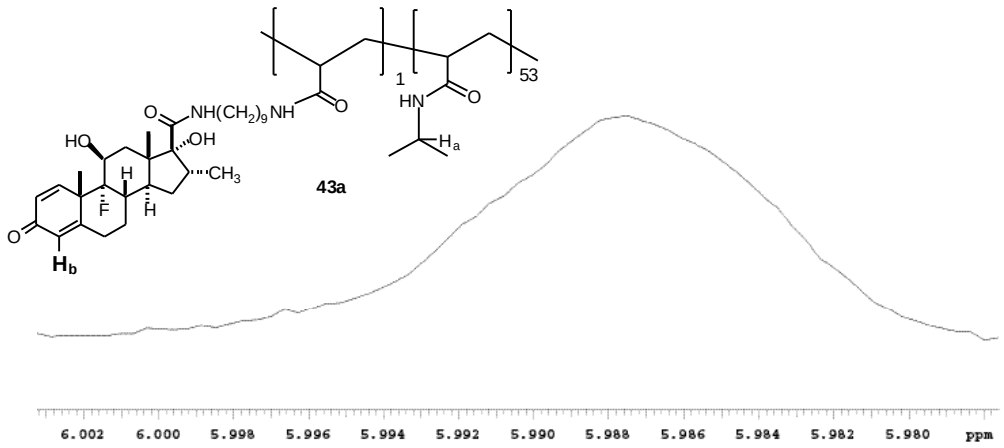
Figure 7. Structure, water solubility data, and ^{1}H NMR (500 MHz, DMSO-d_6) spectra of dexamethasone-polymer macroligand **43a-d**. (a) structure and water solubility of dexamethasone-macroligands synthesized; (b) expansion of ^{1}H NMR (δ 3.5-6.5) spectra showing relative ratio of ligand to polymer based on relative integration of H_a and H_b

In order to compare affinity precipitation with traditional affinity chromatography, we also synthesized the dexamethasone-linker-biotin complex **51** (Scheme 9).

Scheme 9



We explored the application of macroligand **43** for isolation of the glucocorticoid receptor from rat liver, however, we were unable to isolate the glucocorticoid receptors. We considered the possibility that the problem may stem from unsuccessful covalent attachment of dexamethasone ligand to the polymer. We first tried to use thin-layer chromatography (TLC) to see if the free ligand was simply trapped in the polymer. The mobility of the polymer on TLC obscured these results : In none polar solvents, the polymer and free ligand remained at the baseline. When more polar solvents were employed, the polymer always streaked, thus masking everything under it. Ultimately, we found some evidence for covalent attachment by ^{1}H NMR line broading (Figure 8).



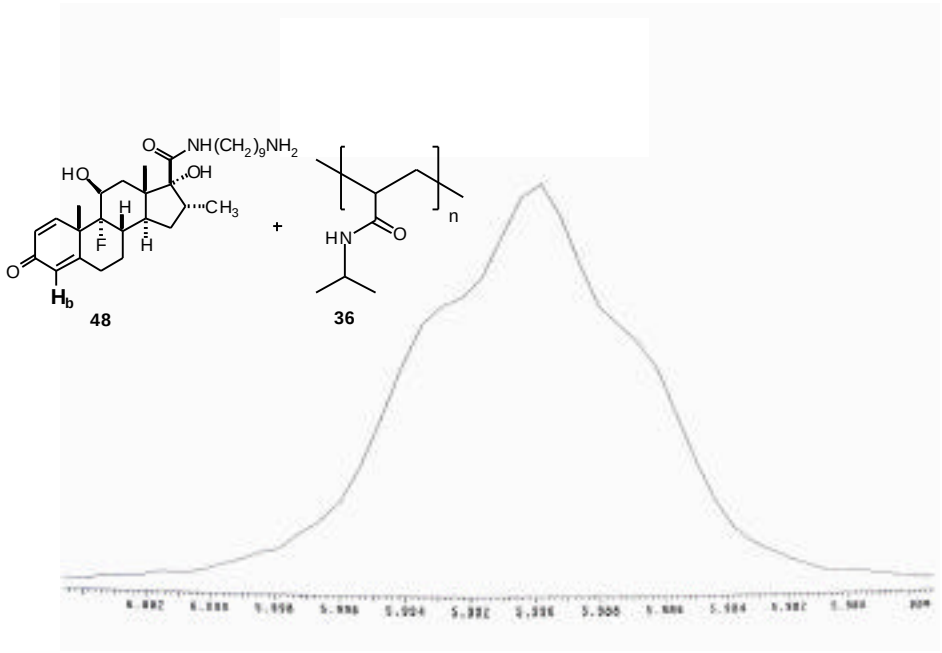


Figure 8. Comparison of width at half-height in ^{1}H NMR (500 MHz, DMSO-d_6) of H_b in (a) macroligand **43c** ($w_{1/2}$ = 4.55 Hz) versus (b) mixture of dexamethasone amine **48** and PNIPAM **36** ($w_{1/2}$ = 4.12 Hz)

As can be seen there is an increase of 0.43 Hz in the signal corresponding to H_b in macroligand **43a**, suggestive of attachment resulting in line broadening due to restricted tumbling. To ensure our ability to isolate the glucocorticoid receptors by traditional affinity methods, we used the biotin-dexamethasone conjugate **51** to isolate dexamethasone receptors from the same batches of rat liver cytosol used in the previous affinity experiment with the dexamethasone macroligand. The failure to isolate the receptors by traditional methods revealed that the previous unsuccessful affinity experiment using dexamethasone-polymer complex may stem from the rat liver cytosol preparation. In addition, it is known that the concentration of glucocorticoid receptors in normal rat liver is very low,[47,50] and traditional purification methods typically used adrenalectomized rats which induces overexpression of glucocorticoid receptors to facilitate the isolation procedure. However, we were unable to obtain adrenalectomized rats. Furthermore, the glucocorticoid receptor is very sensitive to temperature and proteases.[36] Thus, we concluded that the glucocorticoid receptor may not be an ideal proof of principle system. However, we recognized that it is a potentially ideal future candidate to test whether the polymer affinity system can be applied to the isolation of sensitive receptors.

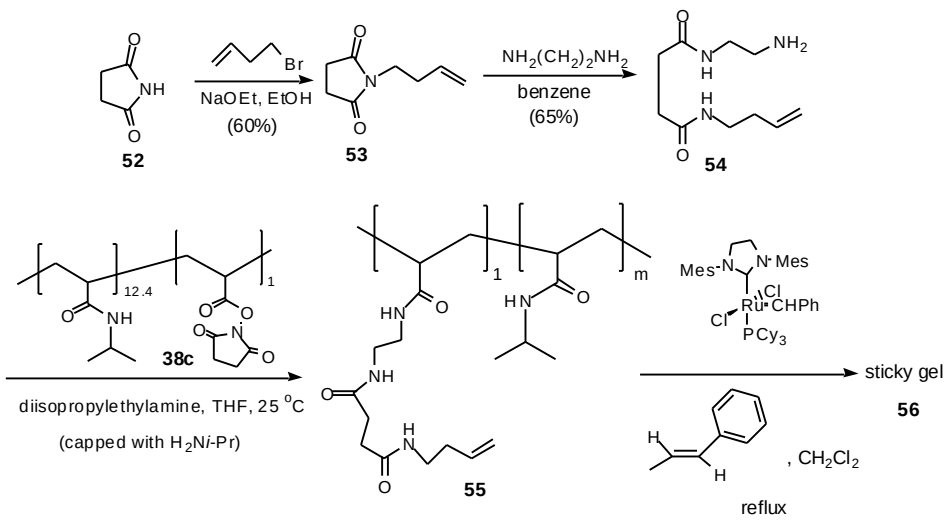
3. Applying a Cyclosporin A-polymer Macroligand to Isolate Cyclophilins

The unique pharmacological profile of cyclosporin A (CsA) as an immunosuppressive drug has led to great interest from both chemists and biologists. Cyclosporin A is currently used clinically to prevent organ rejection in transplant

patients. When CsA traverses the cell membrane, it becomes tightly bound by a cytosolic protein cyclophilin A.[29,38] The CsA-cyclophilin A complex then binds to and inhibits the protein phosphatase activity of calcineurin, an essential mediator of calcium signaling in T cells, affecting cellular signaling.[39,40,41] Cyclophilins not only contain a superfamily of cellular proteins with peptidyl-prolyl isomerase activity, but also have an effect in diverse cellular processes.[42,43] Due to the abundance of several known cyclophilins (e.g. 18, 19, 40, 58, 60, 80, 95, 180 kD) and their known stability,[37a,39] we decided to use CsA-macroligand for our second proof of principle studies.

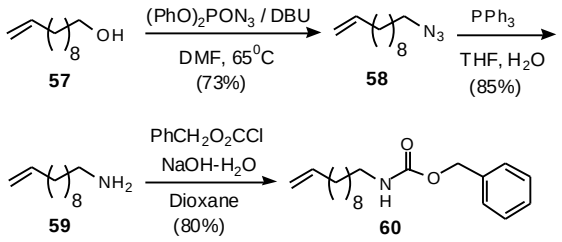
We initially sought to connect the polymer to a linker bearing a terminal alkene. Exploiting olefin metathesis as recently described by Diver and Liu,[37a] the terminal olefin in the polymer would then serve as a point of attachment of CsA. Importantly, this strategy might be generally applicable for attachment of other alkene-modified natural products. Alkylation of succinimide **52** gave imide **53**,[12] which was subsequently treated with 1,2-ethanediamine to give the linker diamide **54** (Scheme 10).[13] Coupling of diamide **54** and PNIPAM **38c** utilizing previously established conditions, afforded polymer-ligand complex **55**. We next turned our attention to the olefin metathesis reaction using Grubbs' second generation ruthenium catalyst.[37] We used trans-β-methylstyrene as a model olefin for the cross coupling prior to the use of CsA.[14] However, it appeared that cross-linking of the polymer was faster than coupling to styrene, since the final product formed was a sticky gel-like material that was not soluble in many common organic solvents unlike the parent polymer. This precluded analysis and characterization by ^{1}H NMR.

Scheme 10



In order to attach CsA to the polymer, a suitable linker had to be synthesized. Synthesis of a protected linker began with the ω-undecynl alcohol (**57**). The alcohol was transformed into azide **58** under Mitsunobu conditions (Scheme 11).[52] Reduction of the azide moiety under Lindlar reduction conditions resulted in simultaneous reduction of the alkene. Chemoselective reduction of the azide **58** was successful upon exposure to triphenylphosphine in wet THF, releasing the primary amine **59**. Finally, the primary amine was masked as the Cbz-carbamate **60** under standard conditions.

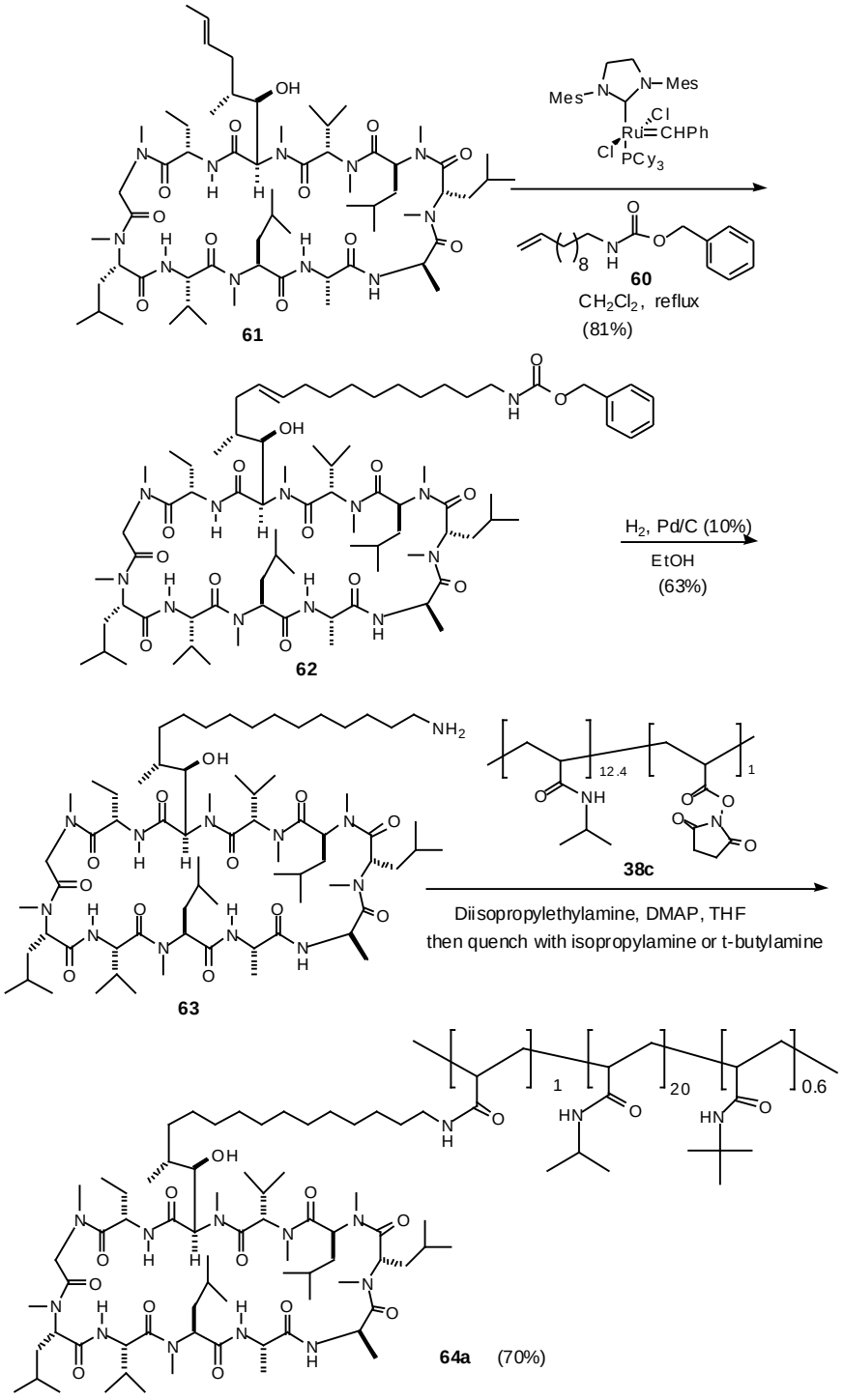
Scheme 11



We next began exploring the olefin metathesis reaction between CsA **61** and amine **60** (Scheme 12). However, the cross-coupling reaction did not go to completion even in the presence of excess amine **60**. Because the adduct **62** cannot be separated from the starting cyclosporin A by column chromatography, a small amount of CsA was sacrificed during the next deprotection step. Removal of the Cbz-protecting group from CsA derivative **62** led to amine **63**, which differs significantly in TLC behavior from CsA, thus allowing chromatographic separation.

During the formation of the macroligand **64a**, we need to deal with the following several conditions carefully: 1) Since the product **62** was relatively polar, it was not very easy to get absolutely clean **62** without the contamination by some yellow byproduct. Although a second column chromatography could be used to improve the purity of the product **62**, the yield of the reaction would be decreased. Later we found that if continue the next reaction without considering the contamination, we can get rid of the color finally. 2) When **62** was exposed to ethanol too long during the deprotection, *N*-ethylated **63** was obtained. So it is better to stop the reaction as soon as it finishes. 3) The purification process to get final macroligand **64a** was not too easy. During the repetitive precipitation, the tiny power-like **64a** often clung to the filter funnel. When **64a** was dry, it was too light to transfer to the flask. The best method to solve this problem was to transfer **64a** during wet condition to the flask. Then connect the flask to high vacuum to remove the excess solvent.

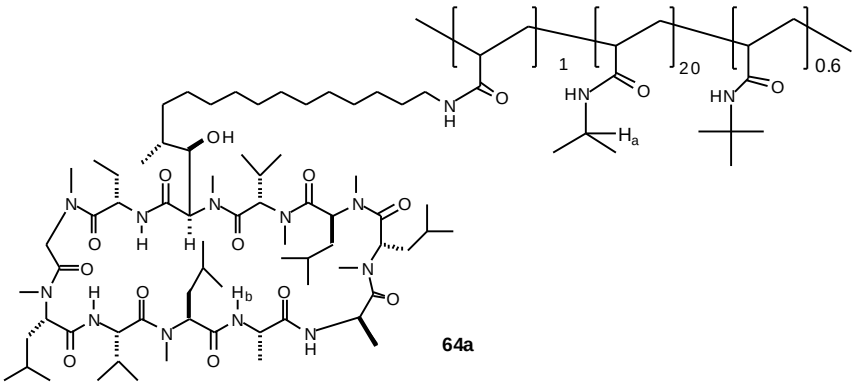
Scheme 12


61
60
CH_2Cl_2, reflux
(81%)
62
H_2, Pd/C (10%)
EtOH
(63%)
63
38c
Diisopropylethylamine, DMAP, THF
then quench with isopropylamine or t-butylamine
64a (70%)

As we required low levels of CsA loading, measurement of the loading became difficult as it approached the detection limits of the ^{1}H NMR. Furthermore, unlike the dexamethasone-polymer complex, which has a diagnostic and well resolved olefinic proton signal to use for integration, CsA does not have any characteristic peaks for integration. We finally chose one of the CsA amide peaks in the proton NMR as a fingerprint for CsA. However the relative magnitude of this peak became very low at low loadings, thus allowing for a greater degree of uncertainty in measurements of loading by integration. Adding to these difficulties is the poor quality of the proton NMR spectra of CsA in DMSO-d_6. As a result of these problems, we could only make an approximation about the CsA loading by ^{1}H NMR spectroscopy in deuterated chloroform (Figure 9).

Alternatively, we also considered several other methods to synthesize cyclosporin A derivative with a terminal amine. However, each of these methods had limitations. As a first approach, we were considered an olefin metathesis between the CsA and ω-undecylenyl azide **58**. Unfortunately, the yield of this reaction is very low. A second strategy was use of an *N*-phthalimide protected amine to cross-metathesize with CsA. Finally, a deprotection of the imide would produce the desired CSA-linked amine. Much to our surprise, the olefin metathesis between CsA and *N*-phthalimide led to a mixture of products with a broad (M-14, M-28, M-42, M+14) mass range suggestive of degradation by cross metathesis of the starting substrates.

a) CsA Macroligand Structure



b) ^{1}H NMR spectra of **64a**

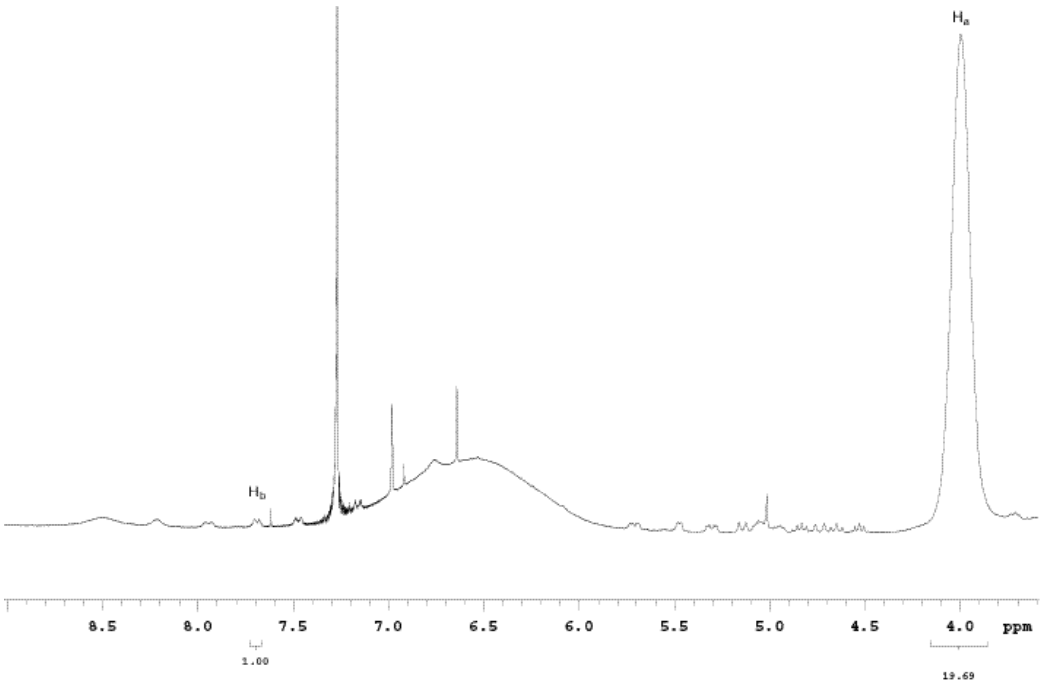
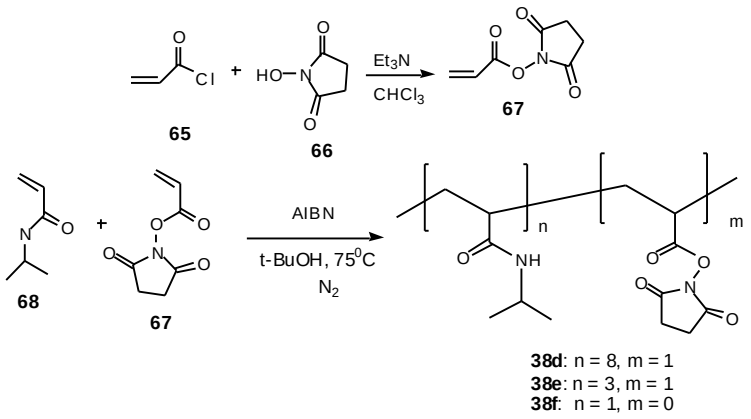


Figure 9. Structure and ^{1}H NMR spectra of **64a**

Critical to the success of this project was the determination of the appropriate amounts of a ligand required to ensure precipitation of the polymer around 30 °C or less. To address these issues, we collaborated with Dr. Bergbreiter's group for the synthesis of polymers **38d**, **38e**, **38f** (Scheme 13). These three polymers showed reasonable water solubility under certain conditions. We found that benzylamine had a remarkable effect on the solubility of these polymers. Trace amounts of benzylamine, attached to polymers **38d**, **38e**, **38f** after amide formation were significant and prevented dissolution even at 4 °C.

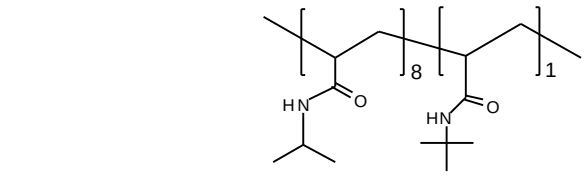
Scheme 13



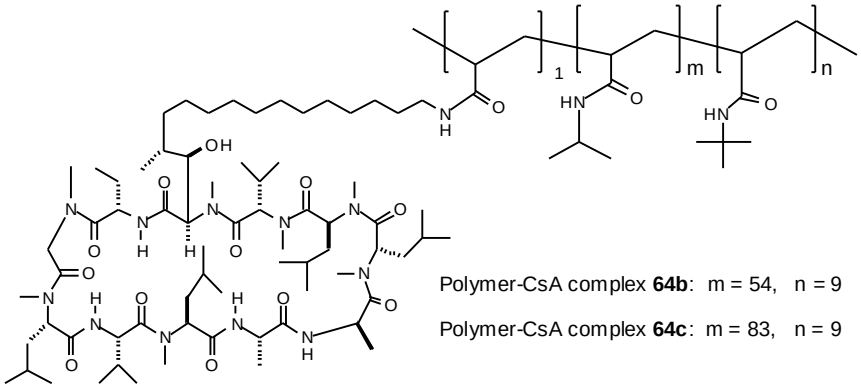
We began affinity chromatography experiments with macroligand **64a** and the traditional Sepharose-CsA beads concurrently (Figure 10). With Sepharose-CsA, we could see one clear band with a molecular weight of approximate 18 kD. In the case of the CsA macroligand, we could also see a similar 18 kD band but with less intensity. At this point, we cannot definitely conclude that this band is a cyclophilin since the poor solubility of free CsA in buffer at 4 °C prevented us from running a true competition experiment. In addition, we found that the polymer-CsA was more readily precipitated out of the buffer solution than the polymer-*t*-butylamine (control) after the affinity binding. Furthermore, the polymer **38d** (8:1 ratio quenched with *t*-butylamine)

precipitated out as small loose solid that prevented centrifugation.

a)



Polymer background **38g**



Polymer-CsA complex **64b**: m = 54, n = 9

Polymer-CsA complex **64c**: m = 83, n = 9

b)

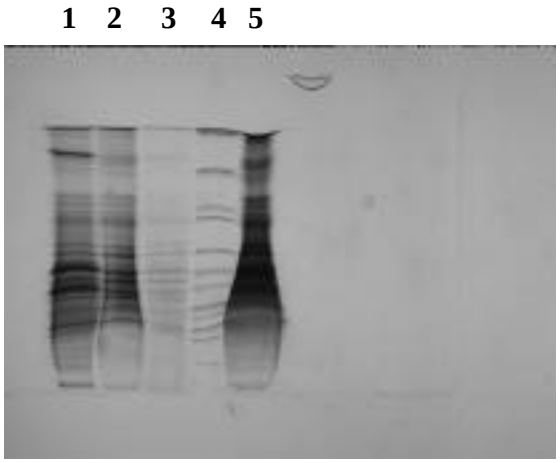
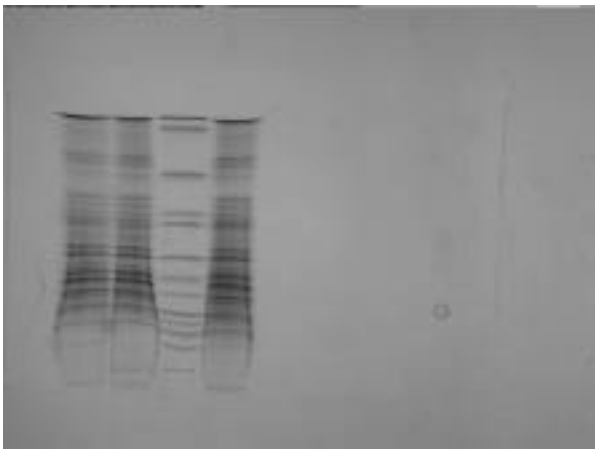


Figure 10. Affinity chromatography experiments using CsA macroligands to isolate cyclophilins from Jurkat cells. a) structures of CsA macroligands **64b** and **64c** used in affinity experiments. b) SDS-PAGE gel from attempted affinity experiment to isolate cyclophilins: Lane 1: Sepharose-CsA complex; Lane 2: Polymer background **38g**; Lane 3: CsA macroligand **64b**; Lane 4: Molecular Weight Marker (6.5-20.5 kDa); Lane 5: CsA macroligand **64c**

One of the challenges during this affinity experiment was the fact that the polymer-CsA macroligand itself cannot be easily precipitated out once the SDS sample buffer was added to the system. This would suggest that the final protein-SDS buffer mixture contains most of the CsA-macroligand. After separation by gel electrophoresis and stained by Coomassie Blue, the bands seemed a little curved and broadened due to the effect of the remaining polymer. We tried several methods including filtration, heating, and changing monomer ratio to cancer out the polymer background. Among these methods, the most effective one we have found was that after adding the SDS sample buffer, heating the total solution in boiling water for 7 min and then centrifuge at 14000 rpm, 34 °C for 5 min. Once the solid was discarded, the liquid was subjected to this heating-centrifugation cycle two additional times. By this method, we were able to remove most of the polymer-macroligand from the protein solution and abtain cleaner gels (Figure 11). The next goal of this research will be to use sangliferin A to perform a competition experiment to ensure that the 18 kD band came from specific binding instead of nonspecific trapping in the matrix.

In the future, sanglifehrin A will be used in competition experiments due to its greater solubility. If the cyclosporin A macroligand enables isolation of the cyclophilins, it will be useful to reinvestigate the dexamethasone macroligand to determine if this methodology can be employed to purify sensitive receptors.

1 2 3 4



1: Polymer-CsA complex **64c** (washed one time); 2: Same with 1 except adding free CsA for competition (Free CsA not dissolve at 4 °C); 3: Molecular Marker; 4: Initial preclearing of the cell lysate with polymer background, then identical to 1

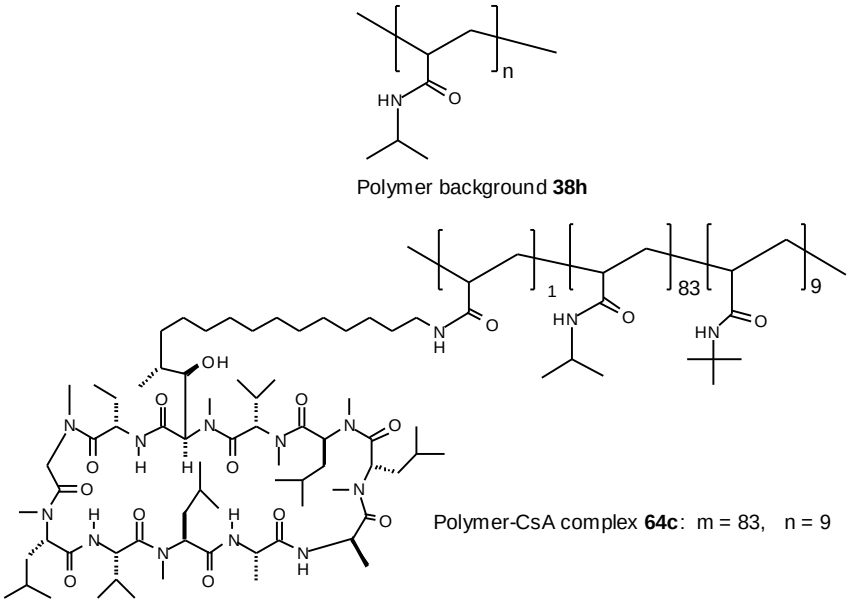


Figure 11. Affinity gel for attempted cyclophilin isolation

CHAPTER V

CONCLUSION

Two natural products dexamethasone **45** and cyclosporin A **61** have linked to a PNIPAM polymer **38** to provide macroligands for affinity chromatography. Analysis of these macroligands by ^{1}H NMR allowed us to determine approximate ligand loadings by intergration. We provided evidence for covalent attachment of dexamethasone and the polymer by measuring ^{1}H NMR line broadening in attached dexamethasone. We also found it simple to determine the ligand loading for the dexamethasone macroligand but not the cyclosporin A macroligand since the former has a very highly characteristic vinyl proton. We found, as expected, that if the ligand loading is relatively low, the macroligand shows similar water solubility to the mother polymer. It dissolved very well in water at 4 °C and precipitated around 35 °C. The dexamethasone polymer macroligand, also indicated that if the ligand is very hydrophobic and loading is relatively high, the macroligand cannot dissolve well at 4 °C. We also attempted initial studies toward use of these macroligands to purify their respective receptors. In the case of the dexamethasone affinity experiment, we did not get the desired receptors partly due to the instability of the glucocorticoid receptor. We also conducted affinity experiments with a cyclosporin A macroligand. While we have not definitely determined if the desired cyclophilins were isolated due to the lack of an effective competition experiment, we determined an effective method to clear the polymer before applying to gel electrophoresis.

CHAPTER VI

EXPERIMENTAL PROCEDURES

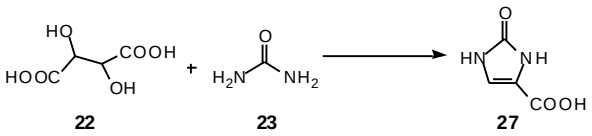
1. General

All non-aqueous reactions were carried out under nitrogen atmosphere in oven-dried (120 °C) glassware unless noted otherwise. Tetrahydrofuran (THF) and diethyl ether (Et_2O) were distilled immediately prior to use from sodium metal/benzophenone ketyl. Methylene chloride (CH_2Cl_2, EM Science) and benzene (EM Science) were distilled from calcium hydride prior to use. Methanol (MeOH, EM Science) was distilled from magnesium methoxide. Triethylamine (Et_3N, EM Science), 2,6-lutidine (Acros) and pyridine (py., EM Science) and diisopropylamine (EM Science) were distilled from calcium hydride immediately prior to use. The molarities indicated for organolithium reagents were established by titration with 2,6-di-tert-butyl-4-methylphenol and 1,10-phenanthroline as the indicator. All other commercially obtained reagents were used as received. Brine refers to a saturated aqueous solution of sodium chloride. Rochelle's salt solution refers to 2 M aqueous sodium potassium tartrate.

Infrared spectra were recorded with a Nicolet Impact 410 FTIR spectrometer. ^1H NMR and ^{13}C NMR spectra were recorded on an Inova-500, Unity-300, VXR-300, or spectrometer. ^1H NMR chemical shifts are reported as δ values in ppm relative to tetramethylsilane (TMS, 0.00 ppm), residual $CHCl_3$ (7.24 ppm), residue acetone (2.05 ppm) or residual C_6H_6 (7.16 ppm). ^1H NMR coupling constants (J) are reported in Hertz (Hz), and multiplicities are indicated as follows: s (singlet), d (doublet), t (triplet), q

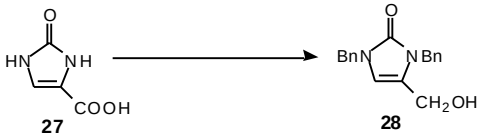
(quartet), quint (quintet), m (multiplet), br s (broad singlet), app (apparent), dd (doublet of doublets). Unless otherwise indicated, deuterochloroform ($CDCl_3$, 77.0 ppm) served as an internal standard for all ^{13}C spectra. Flash column chromatography was performed using 60Å Silica Gel (EM Science, 230-400 mesh) as a stationary phase as described by Still.[1] Mass spectra were obtained on a VG analytical 70S high resolution, double focusing, sectored (EB) mass spectrometer at the center for Chemical Characterization and Analysis (Texas A&M). Thin layer chromatography (TLC) was performed using glass-backed silica gel $60F_{254}$ (EM Science, 250 μm thickness). Cyclosporin A was purchased from LC Laboratories. The *N*-Hydroxysuccinimido Biotin was purchased from Sigma. Dexamethasone-Sepharose was obtained from Professor Jun Liu's lab (John Hopkins University). Cyclosporin A-Sepharose was initially obtained from Professor Jun Liu's lab. It was subsequently synthesized according to the method of him.[37] Electrophoresis was performed using Mini-Protean 3 Cell Assembly (Bio-Rad). The gel was rinsed by shaking with The Belly Dancer (Stovall Life Science).

2. Procedures



22 23 27

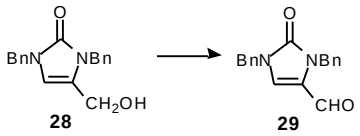
4-Carboxyimidazolin-2-one 27: To 660 mL H_2SO_4 (18 M) was slowly added a mixture of tartaric acid (**22**) (272.1 g, 1.8 mol) and urea (**23**) (100.0 g, 1.7 mol). The temperature was not allowed to exceed 65 °C without external cooling. Reaction mixture was heated to 80 °C for 24 h. The dark brown reaction mixture was poured over ice (3 kg) resulting in the precipitation of a brown solid. The precipitate thus obtained was isolated by vacuum filtration and washed with H_2O and acetone to give 73.2 g (45%) of 4-carboxyimidazolin-2-one **27** as a brown solid. Spectral data for this compound matched that previously reported.[45]



27 28

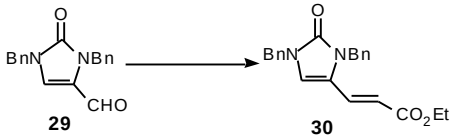
1,3-Bis(benzyl)-4-(hydroxymethylene)imidazol-2-one 28: To a cooled (0 °C) suspension of NaH (6.51 g, 270.7 mmol) in 170 mL anhydrous DMF was added 4-carboxyimidazolin-2-one **27** (7.81 g, 60.16 mmol) and Bu_4NI (4.42 g, 12.03 mmol). The reaction mixture was warmed to 25 °C. After stirring for 30 minutes, benzylbromide

(28.6 mL, 240.63 mmol) was added dropwise (4 mL/h) by syringe pump. After stirring for 10 h at 90 °C, the reaction was carefully quenched by addition H_2O (10 mL). The mixture was extracted with Et_2O. The combined organic layers were washed with water and brine, dried over anhydrous $MgSO_4$ and concentrated in *vacu*o. Using distillation to evaporate the benzylalcohol, benzylether, benzylbromide to get 5.87 g(crude) tribenzyl-4-carboxyimidazolin-2-one. The crude ester in 113 mL CH_2Cl_2 was cooled to –78 °C. DIBAl-H in CH_2Cl_2 (44.3 mL, 2 M) was added to the ester solution. After stirring for 3 h at –78 °C, a solution of Rochelle's salt was added and the heterogeneous mixture was allowed to warm to 24 °C for 12 h. The layers were separated and the aqueous layer was extracted with CH_2Cl_2. The combined organics were washed with brine, dried over anhydrous Na_2SO_4 and concentrated in *vacuo.* Purification by flash chromatography on SiO_2 eluting with hexane:EtOAc(10:1→1:10) gave alcohol **28** (2.52 g, 36.3% for 2 steps) as a yale solid. Spectral data for this compound matched that previously reported.[45]

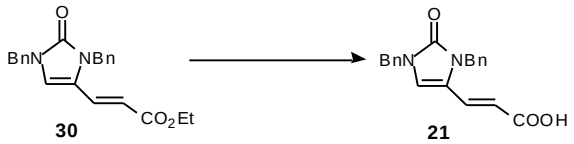


1,3-Bis(benzyl)4-(carboxaldehyde)imidazol-2-one 29: To activated MnO_2 (8.0 g, 92 mmol) was added 4-hydroxymethylimidazolin-2-one **28** (868.8 mg, 2.95 mmol) as a solution in 12 mL CH_2Cl_2, rinsing twice with using 5 mL CH_2Cl_2 to complete the transfer. After stirring for 4 h at ambient temperature, the reaction mixture was filtered

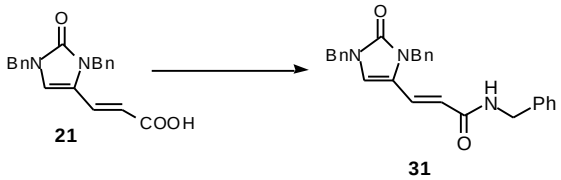
through Celite and concentrated in *vacuo* to give aldehyde **29** (0.7144 g, 83%) as a yellow oil. No further purification was required. Spectral data for this compound matched that previously reported.[45]



α,β-unsaturated Ester 30: To a cooled (0 °C) suspension of NaH (111.4 mg, 4.64 mmol) in 10 mL of THF was added triethylphosphonoacetate(0.73 mL, 3.68 mmol) in 10 mL (8 mL rinse) THF. After stirring the reaction at 0 °C for 5 min, the reaction mixture was warmed to 24 °C amd stirred for 1 h. A solution of aldehyde **29** (710 mg, 2.43 mmol) was slowly added in 7 mL (5 mL rinse) THF. After stirring for 2 h, the reaction mixture was poured over 10 mL of water. The layers were seperated and the aqueous layer was extracted with Et_2O. The combined organics were washed with brine, dried over anhydrous Na_2SO_4 and concentrated in *vacuo.* Purification by flash chromatography on SiO_2 eluting with hexane:EtOAc (10:1→1:5) gave α,β-unsaturated ester **30** (527.7 mg, 60%) as a yellow solid. Spectral data for this compound matched that previously reported.[45]

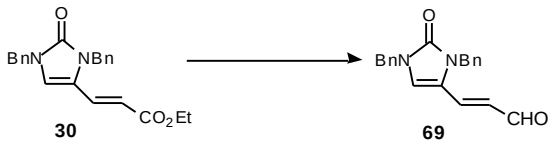


α,β-unsaturated Acid 21: To a cooled (0 °C) solution of α,β-unsaturated ester **30** (63.9 mg, 0.18 mmol) in 2 mL THF was added LiOH (1 M, 2.8 mL) in a dropwise manner. After 0.5 h , the cold bath was removed and the mixture was stirred at 24 °C for 6 h. The reaction was quenched by adding dilute 1N HCl until pH paper indicated pH 1-2 and the layers were separated. The aqueous layer was extracted four times with 8 mL EtOAc, and the combined organic layers were dried over anhydrous Na_2SO_4 and concentrated in *vacuo* to yield acid **21** as a white solid (58.9mg, 99%) in nearly quantitative yield: R_f = 0.35 (1:3-hexane:EtOAc); IR ($CHCl_3$) 3012, 2914, 1685, 1624, 1455 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.50-7.20 (m, 10H), 7.18 (d, *J* = 16 Hz, 1H), 6.60 (s, 1H), 5.91 (d, *J* = 16 Hz, 1H), 5.05 (s, 2H), 4.87 (s, 2H); ^{13}C NMR (500 Mhz, $CDCl_3$) δ 170.8, 153.7, 136.4, 135.7, 131.9, 129.0, 128.9, 128.3, 128.1, 127.7, 126.7, 120.1, 115.6, 113.2, 47.7, 45.4; HRMS (ESI) Calcd for $C_{20}H_{18}N_2O_3$ [M+H]: 335.1317. Found: 335.1409



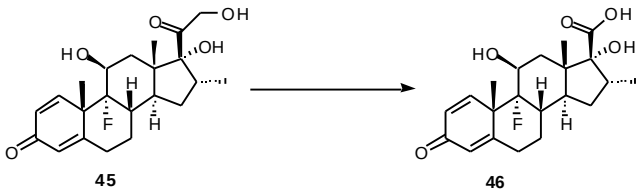
α,β-unsaturated Amide 31: To a cooled (-78 °C) solution of acid **21** (205 mg, 0.614 mmol) in 4.5 mL THF was added Et_3N 85.42 μL . Five minutes later,

isobutylchloroformate (76.64 μL, 0.614 mmol) was added dropwisely to the solution. After stirring under –78 °C for 10 minutes, the reaction temperature was raised to –20 °C. And after 0.5 h, a solution of benzylamine (87.0 μL) and Et_3N (85.4 μL), in THF (2.5 mL) was added to the reaction. Stirring was continued for an hour, the solvent was evaporated, and 50 mL EtOAc was added to the reaction residue. The solution was washed with brine, dried over Na_2SO_4, and concentrated in *vacuo*. Purification by flash chromatography on SiO_2 eluting with hexane:EtOAc (10:1→1:4) gave Benzylamide **31** (0.14 g, 52%) as a yellow viscous oil : R_f = 0.29 (1:3-hexane:EtOAc); IR ($CHCl_3$) 3283, 3064, 2925, 1666, 1451 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.39-7.21 (m, 16H), 6.53 (s, 1H), 5.90 (d, *J* = 15.6 Hz, 1H), 5.67 (t, *J* = 5.7 Hz, 1H), 5.04 (s, 2H), 4.88 (s, 2H), 4.60 (d, *J* = 5.7 Hz, 2H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 128.9, 128.8, 128.7, 128.0, 127.8, 127.6, 126.7, 47.5, 45.3; HRMS (ESI) Calcd for $C_{27}H_{25}N_3O_2$ [M+H]: 424.1947. Found: 424.2002.



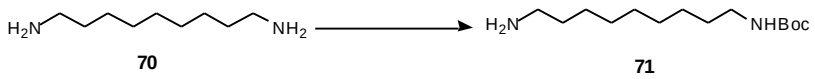
α,β-unsaturated Aldehyde 69: To a cooled (-78 °C) solution of ester **30** (212 mg, 0.59 mmol) in 7 mL anhydrous CH_2Cl_2 was added a 2 M solution of DIBAl-H in CH_2Cl_2 (1.76 mL, 3.5 mmol). The reaction was stirred at -78 °C for 15 min, then diluted with 10 mL CH_2Cl_2 followed by adding addition of 9.44 g $Na_2SO_4 \cdot 10H_2O$. After vigorous

stirring for 2 h, the reaction mixture was filtered through celite and concentrated in *vacuo* to provide crude alcohol (187.6 mg, 0.59 mmol). To activated (flame dry under high vacuum three times, every time 1 minute) MnO_2 (2 g, 23.44 mmol) was added the above crude alcohol as a solution in 8 mL (5 mL rinse) CH_2Cl_2. After stirring for 3 h at 24 °C, the reaction mixture was filtered through celite and concentrated in *vacuo.* Purification by flash chromatography on SiO_2 eluting with hexane: EtOAc system (10:1→1:5) gave of aldehyde **69** (100.3 mg, 54%) as a white solid: R_f = 0.62 (3:7-hexane:EtOAc); ^{1}H NMR (300MHz, $CDCl_3$) δ 9.35 (d, *J* = 7.8Hz, 1H), 7.40-7.19 (m, 10H), 6.86 (dd, *J* = 15.9 Hz, 0.6 Hz, 1H), 6.74 (s, 1H), 6.19 (ddd, *J* =15.9 Hz, 7.5 Hz, 0.5 Hz, 1H), 5.08 (s, 2H), 4.92 (s, 2H); ^{13}C NMR (300 MHz, $CDCl_3$) δ 192.5, 137.0, 136.3, 135.6, 132.1, 132.0, 129.1, 129.0, 128.6, 128.4, 128.2, 127.8, 126.5, 124.6, 120.3, 116.6, 124.6, 120.3, 116.6, 47.8; HRMS (ESI) Calcd for $C_{20}H_{18}N_2O_2$ [M+H]: 319.1368. Found: 319.0000



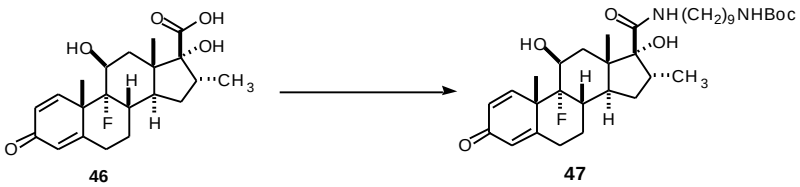
Dexamethasone acid 46: To a solution of dexamethasone **45** (1.0 g, 2.55 mmol) in 200 mL of ethyl alcohol was added a solution of periodic acid (120 mL, 0.025 M) and sulfuric acid (3.8 mL, 2.5 M) in 75 mL distilled water. After the reaction mixture was stirred for 24 h at 24 °C, the ethanol was evaporated in *vacuo,* and the residue was

filtered to get a crude solid. The solid was dissolved in ethylacetate (200 mL) and extracted three times with NaOH (0.04 M, 40 mL). The combined aqueous layers were then acidified to pH = 2 with 2M HCl. This solution was aged at 24 °C. Filtration of the resulting solids gave acid **46** (0.675 g, 70 %) as a white solid. Spectroscopic data for this compound matched that previously reported.[34]



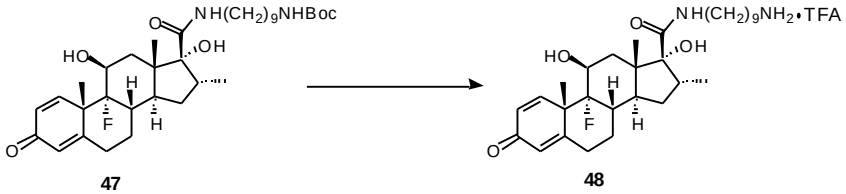
70 **71**

(9-Amino-nonyl)-carbamic acid *tert*-butyl ester 71: To a solution of diamine **70** (1.0 g, 6.3 mmol) in 5 mL THF at 24 °C was added a solution of $(Boc)_2O$ (0.69 g, 3.2 mmol) in THF dropwise. After stirring overnight, the solvent was removed in *vacuo*, and 6 ml of water was added. The solution was extracted with CH_2Cl_2 (3 x 6 mL). The combined organic extracts were then washed with brine (4 x 6 mL) and dried over anhydrous $MgSO_4$. Filtered and concentrated. Purification by flash chromatography on SiO_2 eluting with EtOAc: MeOH: Et_3N = 10:2:1 afforded mono-*N*-Bocdiamine **71** (0.82 g, 99 %). Spectroscopic data for this compound matched that previously reported.[46]

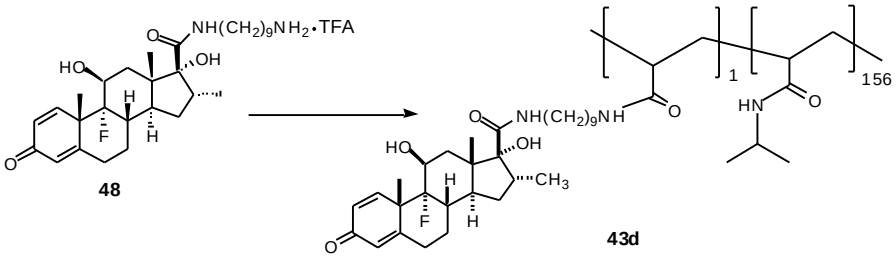


46 **47**

Dexamethasone amide 47: To a solution of dexamethasone acid (234.6 mg, 0.62 mmol), (9-Amino-nonyl)-carbamic acid *tert*-butyl ester **71** (349.8 mg, 1.4 mmol), HOBt (162.44 mg, 1.24 mmol) in 35 mL anhydrous CH_2Cl_2 was added Et_3N (0.81 mL, 5.83 mmol). After 5 min, EDCI (498.4 mg, 2.6 mmol) in 25 mL CH_2Cl_2 was transferred to the reaction mixture. After stirring for 5 h at 24 °C, the reaction mixture was diluted with EtOAc (250 mL) and washed with 0.1M HCl (3 x 55mL), saturated $NaHCO_3$ (1 x 55mL), water (1 x 55mL), and then dried over Na_2SO_4. Purification by flash chromatography on SiO_2 eluting with Et_2O:EtOAc(19:1→1:1) gave the Bocprotected amide **47** (318.9 mg, 80%) as a white solid: R_f = 0.59 (1:2-Et_2O:EtOAc); ^1H NMR (300 MHz, $CDCl_3$) δ 7.24 (s, 1H), 6.60 (t, *J* = 4.6 Hz, 1H), 6.36 (dd, *J* = 10.3 Hz, 2.0 Hz, 1H), 6.15 (s, 1H), 4.60 (bs, 1H), 4.39 (d, *J* = 9.3 Hz, 1H), 3.25 (m, 6H), 2.66 (m, 1H), 2.50-2.10 (m, 7H), 1.90-1.70 (m, 2H), 1.60 (s, 3H), 1.48 (s, 13H), 1.33 (s, 11H), 1.16 (s, 3H), 0.97 (d, *J* = 7.5 Hz, 3H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 186.6, 172.3, 166.2, 152.1, 129.8, 125.1, 86.8, 72.4, 71.8, 47.9, 43.8, 39.3, 36.5, 35.2, 34.4, 34.2, 32.2, 31.1, 29.9, 29.7, 29.2, 28.8, 28.4, 27.4, 26.7, 26.5, 22.9, 17.4, 14.4; HRMS (ESI) Calcd for $C_{35}H_{55}FN_2O_6$ [M+H]: 619.4044. Found: 619.4188

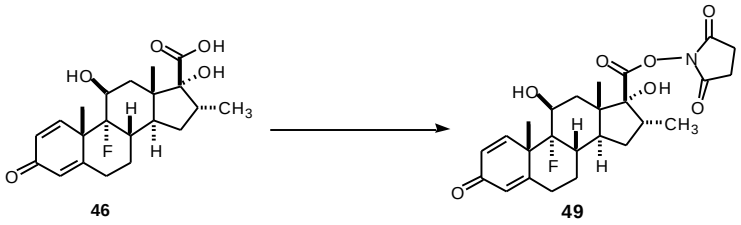


Dexamethasone amine TFA salt 48: To a stirred solution of amine **47** (907.5 mg, 1.5 mmol) in 17.7 mL CH_2Cl_2 was added 11 mL 20% TFA in CH_2Cl_2 solution at 0 °C. After 3 h at 0 °C, the reaction mixture was diluted with 80 mL $CHCl_3$ and then the solvent was evaporated in *vacuo* . Heptane (3 x 10 mL) was added to remove the excess TFA by evaporation to get yellow gel-like amine TFA salt **48** (0.92 g, 99 %). Spectroscopic data for this compound matched that previously reported.[34]

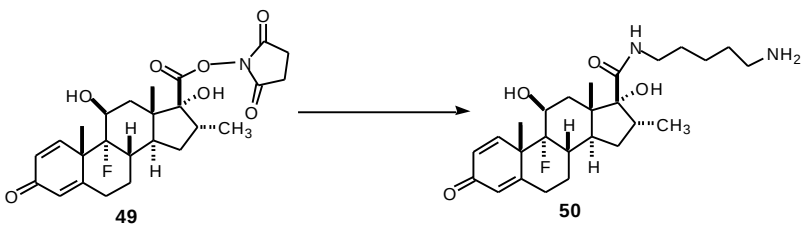


Dexamethasone-polymer macroligand 43d: To a solution of polymer **38c** (227 mg, 0.154 mmol) in 4 mL THF was added a solution of TFA amine salt **48** (9.8 mg, 0.015 mmol), catalytic amount of DMAP, and Hunig's base (4 μL, 0.023 mmol) in 2 mL THF. After 12 h at 24 °C, excess isopropylamine (0.1 mL 1.23 mmol) was added and the reaction mixture was stirred overnight. The reaction mixture was filtered through Celite to remove the *N*-hydroxysuccinimide and the solvent was evaporated in *vacuo*. The crude product was redissolved in 4 mL THF, and the THF solution was added dropwise into 70 mL of rapidly stirring hexane to precipitate the polymer. After repeating this reprecipitation process three times, the solid was isolated by filtration and dried under vacuum to get the product polymer **43d** (205.6 mg, 75%) as a white solid. ^{1}H NMR

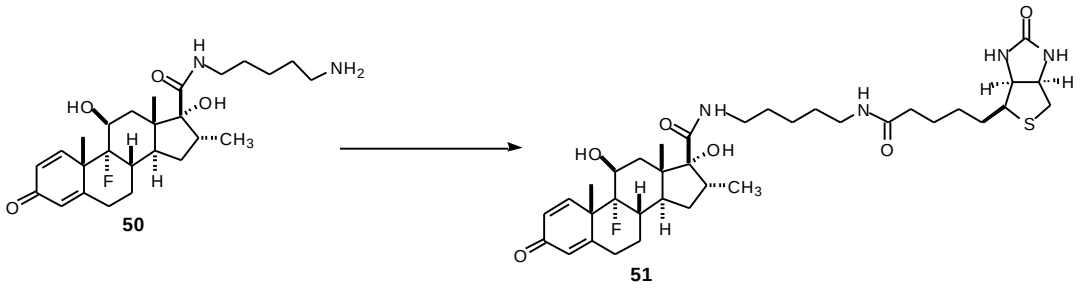
(500 MHz, DMSO-d_6) δ 7.60-7.00 (m, 146H), 6.20 (d, J = 8.3 Hz, 1H), 6.00 (s, 1H), 5.23 (s, 1H), 4.60 (s, 1H), 4.08 (bs, 1H), 3.80 (s, 144H).



Dexmethasonesuccinimiester 49: A solution of dexamethasone acid **46** (85.7 mg, 0.23 mmol), EDCI (67 mg, 0.35 mmol), Et_3N (0.16 mL, 1.15 mmol), *N*-hydroxysuccinimide (132 mg, 1.15 mmol), and HOBt (47 mg, 0.35 mmol) in 22 mL CH_2Cl_2-DMF (10:1) was stirred at 24 °C for 12 h. Then the mixture was poured on HCl (0.1 N, 27 mL) and the layers were separated. The aqueous layer was extracted with EtOAc (3 x 10 mL). After evaporating the solvent, the residue was redissolved in 45 mL EtOAc and washed with 27 mL saturated $NaHCO_3$ and dried over anhydrous Na_2SO_4. Purification by flash chromatography on SiO_2 eluting with hexane:EtOAc(3:1→0:1) gave ester **49** (48.5 mg, 45%) as a white solid. Spectroscopic data for this compound matched that previously reported.[36, 50]



Amide 50: To a solution of diamine (60 μL, 0.52 mmol) in 8.0 mL THF was added dropwise a solution of ester **49** (49.1 mg, 0.10 mmol) in 4.6 mL THF. After 8 h at 24 °C, the solvent was evaporated *in vacuo.* Purification by flash chromatography on SiO_2 eluting with EtOAc: MeOH: Et_3N (1:3:0.2) gave amide **50** (20.4 mg, 43%) as a white solid. Spectroscopic data for this compound matched that previously reported.[36]



Dex-Biotin complex 51: A solution of Amide **50** (20.4 mg, 0.044 mmol), NHS-biotin (22.5 mg, 0.066 mmol) in 1.0 mL DMF was stirred at 24 °C for 14 h. Excess DMF was removed by freeze-drying procedure. Purification of the residue by flash chromatography on SiO_2 eluting with $CHCl_3$: MeOH (12:1→4:1) gave **51** (19.8 mg, 65%) as a white solid. Spectra data for this compound matched that previously reported.[36]

Affinity Chromatography to Isolate the Dexamethasone Receptor from Rat Liver

1) Rat liver cytosol preparation[34,47] : Livers from 40 freshly sacrificed rats were frozen in TMG buffer containing protease inhibitor kit and stored at –20 °C. Prior to use, they

were thawed in water bath (37 °C) for 20 min. All the following procedures were performed at 4 °C. The livers were rinced with TMG buffer containg protease inhibitor kit and homogenized first using a waring blender then with a mortar and pestle. The homogenate was then spun at 35,000 rpm for 2 h to remove lipids and some cellular debris. The supernatant was then brought up to 1% streptomycin sulphate and centrifuged at 10,000 rpm for 15 min to remove the ribonuclear proteins. The supernatant was then brought up to 0.075% protamine sulphate by addition of a 0.75% solution of protamine sulphate drop by drop. The solution was again centrifuged at the same speed and time. The resulting pellet was washed with phosphate buffer and centrifuged at 10,000 rpm for 15 min. This cycle was repeated an additional two times. All washings and the previous supernatant from protamine sulphate precipitation (a total of 105 ml) were used immediately.

2) Affinity experiment for receptor isolation: The following three affinity matrix were prepared: a. 67.2 mg Dex-polymer complex, b. 57.8 mg Dex-polymer complex plus 36.8 mg free dexamethasone acid, c. 61.6 mg polymer fully quenched with isopropylamine. The following representative procedure was the same for the above affinity matrix. Each polymer was mixed with 35 ml of rat liver cytosol solution and shaked at 4 °C for 17 h. The solution was then centrifuged at 11,000 rpm for 10 min at 27 °C. The following procedures were performed at 4 °C. The supernatants were stored and the precipitates were dissolved by the addition of 1.5 mL TMG buffer. All the precipitates dissolved very well under this condition. A solution of dexamethasone acid (25 mg) in 2 mL

TMG buffer was then added dropwise to the above polymer solutions and the resulting solutions were shaken for 12 h. The solutions were centrifuged at 11,000 rpm for 10 min at 27 °C. Both of the precipitates and the supernatants from this step were subjected to SDS-PAGE analysis. The SDS-PAGE gel consisted of: Resolving gel (9%) and Stacking gel (4%). The samples were then loaded on the stacking gel and ran at 10 mA while the proteins were in the stacking gel. When they entered the border between the stacking gel and the resolving gel, 15 mA was applied to the gel. The total running time was around 35 min. The gel was then stained with Coomassie blue and destained to show the protein bands. Unfortunately, we did not see the desired 45 kD and 90 kD bands from the gel.

Analytical Affinity Chromatography with Biotin-Natural Product Conjugates and Soluble Polymer-Dexamethasone Complex[48,49]

Part One : Cell Lysis

The entire procedure was carried out on ice or in a cold room (4 °C)

1. To 32 million cell pellet was added 2.0 mL freeze thaw buffer (10 mM CHAPS).
2. The solution was kept on ice for 10 min.
3. The solution was centrifuged at 14,000 rpm for 25 min. The supernatant was transferred to a clean tube and the pellet was discarded.
4. The protein concentration was measured.

Part Two : Affinity Binding

The entire procedure was carried out in a cold room (4 °C)

1. The lysate was aliquoted into six Eppendorf tubes, 200 µL each. Tube 1 was Setted as DMSO control, tube 2 as Biotin-Dex binding, tube 3 as Biotin-Dex/Dex competition, tube 4 as polymer-Dex binding, tube 5 as polymer-Dex/Dex competition, tube 6 as PNIPAM,
2. DMSO (2 µL) was added to tube 1, DMSO (2 µL) to tube 2, Dexamethasone (2 µL, 1mM) to tube 3, ddH_2O (2 µL) to tube 4, Dexamethasone(20 µL, 1mM) to tube 5, ddH_2O (2 µL) to tube 6. All these tubes were putted on the Belly Dancer in cold room and kept rotating for 0.5 h.
3. DMSO (2 µL) was added to tube 1, Biotin-Dex (2 µL, 100 µM) to tube 2, Biotin-Dex (2 µL) to tube 3, polymer-Dex (15 µL, 2mM) to tube 4, polymer-Dex (15 µL, 2 mM) to tube 5, PNIPAM (15 µL, 2 mM) to tube 6. All these tubes were put on the Belly Dancer in cold room and kept rotating for 1h.
4. Avidin-Sepharose CL-4B was aliquoted to another set of three Ep tubes, 20 µL each. The tubes were spinned down at 8,000 rpm for 2 min. All the supernatant was carefully removed. The beads were washed two times with 200 µL of freeze thaw buffer. Make sure the beads amounts equal. Tubes (1-3) were transferred to the tubes with beads and rotating for 1 h. The tubes (4-6) were rotated for 1 h.
5. For tubes (1-3), the tubes were spinned down at 8,000 rpm for 2 min. The supernatants were removed and the beads were washed with 400 µL of freeze-thaw buffer. The tubes were spinned down again. The beads were washed again and the pellet beads were kept. For tubes (4-6), they were first warmed to 32 °C and then spinned down at 8,000 rpm for 10 min. After the supernatant was removed and the

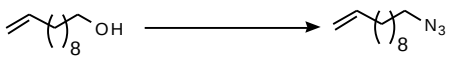
precipitate was redissolved in 200 µL of freeze-thaw buffer, Spin down again and the supernatant was removed and the precipitate was kept.

6. SDS loading buffer (40 µL) was added to each tube. After heated at 100 °C for 10 min, the buffer was spinned down for 5 min at 8,000 rpm.
7. 12 µL of solution was taken from each sample and the SDS-PAGE was run with a protein molecule marker

Part Three: SDS-PAGE

Use 10 % Polyacrylamide Gel

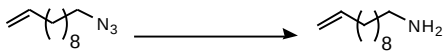
Part Four: Silver Staining



57 58

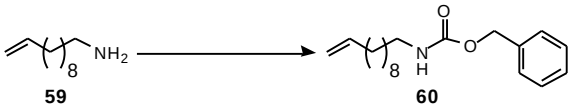
ω-**undecylenyl azide 58**: A mixture of *w*-undecylenyl alcohol **57** (1.2 mL, 6.0 mmol) and Diphenylphosphoryl azide (1.6 mL, 7.3 mmol) was dissolved in anhydrous DMF 10mL. After the mixture was cooled to 0 °C, neat DBU 1.1 mL was added. The reaction mixture was stirred at 0 °C for 2 h and then heated to 65 °C. After the reaction was kept at 65 °C for 5.5 h, EtOAc (50 mL) and H_2O (17 mL) were added and the two layers were separated. The organic phase was washed with H_2O (2 x 10 mL), 5% HCl (10 mL), brine(2 x 10 mL) respectively and dried over anhydrous Mg_2SO_4. Purification by flash

chromatography on SiO_2 eluting with Et_2O:hexane (1:19→1:9) gave ω-undecylenyl Azide **58** (0.83 g, 73%) as a yale solid: R_f = 0.24 (5:1-hexane:EtOAc); IR ($CHCl_3$) 2931, 2851, 2095 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 5.90-5.77 (m, 1H), 5.04-4.90 (m, 2H), 3.25 (t, *J* = 6.9Hz, 2H), 2.10-2.00 (m, 2H), 1.65-1.55 (m, 2H), 1.45-1.25 (m, 12H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 139.0, 114.0, 51.4, 33.8, 29.4, 29.3, 29.1, 29.0, 28.8, 28.8, 26.7.



58 **59**

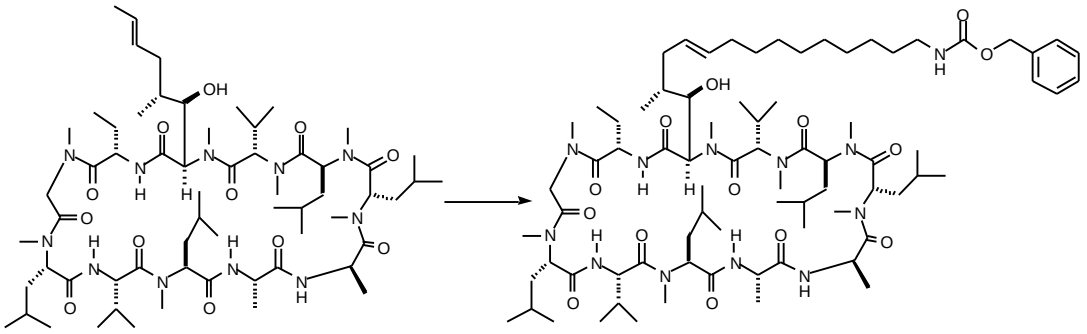
ω-**undecylenyl amine 59:** A mixture of starting Azide **58** (720.0 mg, 3.6 mmol) and triphenylphosphine (1.0 g, 3.9 mmol) in THF 18 mL containing water 0.07 mL was stirred at 24 °C for 18 h and then concentrated in vacuo. Purification by flash chromatography on SiO_2 eluting with CH_2Cl_2:CH_3OH:Et_3N (1:0:0.1→1:10:0.2) to afford *w*-undecylenyl Amine **59** (520.3 mg, 86%) as a yale viscous oil. Spectroscopic data for this compound matched that previously reported.[50]



59 **60**

***N*-Cbz-aminal 60:** To a solution of started Amine **59** (135.9 mg, 0.80 mmol) in 5 mL dioxane was added NaOH (0.29 M, 3 mL) at 0 °C. After the solution was stirred at 0 °C

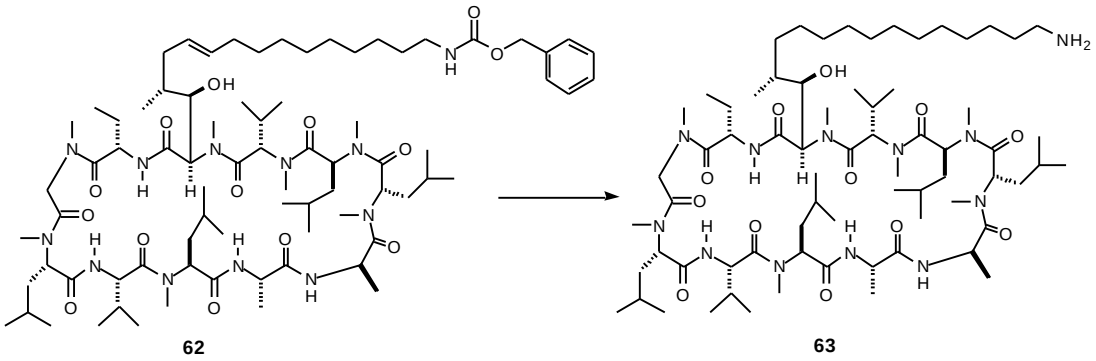
for 15 min, Then Cbz-chloride dioxane solution (1 M, 0.97 mL) was added to the above reaction mixture in a dropwise mode. After the reaction mixture was warmed to 24 °C, the solution was continued stirring for 14 h. Then evaporate the dioxane in vacuo and use dilute hydrochloric acid (1N) to adjust the solution till pH = 3-4. The reaction mixture was extracted with EtOAc (3 x 30 mL). Purification by flash chromatography on SiO_2 eluting with Et_2O:Hexane (1:20→5:1) gave the product **60** (194.9 mg, 80%) as a white solid: R_f = 0.12 (1:3-Et_2O:hexane); IR ($CHCl_3$) 3334, 2919, 1701, 1532, 1255 cm^{-1}; ^{1}HNMR (300 MHz, $CDCl_3$) δ 7.40-7.30 (m, 5H), 5.90-5.75 (m, 1H), 5.10 (s, 2H), 5.05-4.90 (m, 2H), 4.82 (br, 1H), 3.23-3.17 (br, 2H), 2.10-2.00 (m, 2H), 1.60-1.23 (m, 14H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 139.1, 114.1, 45.7, 39.9, 33.8, 31.9, 29.5, 29.4, 29.3, 29.3, 29.3, 29.0, 29.0, 28.9, 28.9, 27.6, 26.5, 22.6, 14.1, 8.6; HRMS (ESI) Calcd for $C_{19}H_{29}NO_2$ [M+H]: 304.2198. Found: 304.2081



61 **62**

Cbz carbamate 62: Into a flame-dried 25 mL round bottom flask equipped with a magnetic stirbar and reflux condensor was dissolved cyclosporine A **61** (209 mg, 0.17

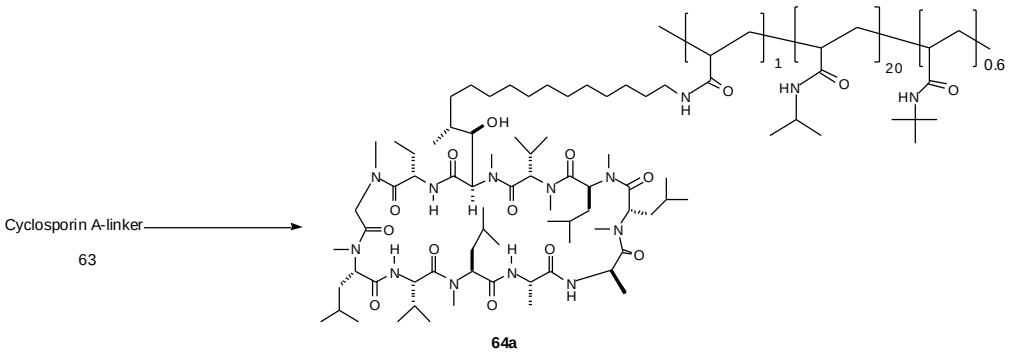
mmol), Cbz protected *w*-undecylenyl Amine (477.6 mg, 1.57 mmol), and 1,3-dimesityl-4,5-dihydroimidazol-2-ylidenetricyclohexylphosphine benzylidene ruthenium dichloride (45 mg, 0.053 mmol, 30 mol%) in 5.0 mL CH_2Cl_2 and the solution was refluxed for 24 h. After cooling to ambient temperature, the reaction mixture was concentrated in *vacuo.* Purification by flash chromatography on SiO_2 eluting with hexane:EtOAc (1:2→1:0) to give **62** (206.6 mg, 81%) as a white solid: R_f = 0.16 (1:5-Et_2O:hexane); IR ($CHCl_3$) 3309, 2995, 2868, 2254, 1629 cm^{-1}; ^{1}HNMR (500 MHz, $CDCl_3$) δ 8.03 (d, *J* = 10.0 Hz, 1H), 7.66 (d, *J* = 7.5Hz, 1H), 7.47 (d, *J* = 8.0 Hz, 1H), 7.38-7.28 (m, 5H), 7.16 (d, *J* = 8.5 Hz, 1H), 7.69 (dd, *J* = 11, 4 Hz, 1H), 5.49 (m, 1H), 5.31 (m, 2H), 5.14-4.92 (m, 6H), 4.82 (m, 1H), 4.71 (d, *J* = 14, 1H), 4.64 (m, 1H), 4.53 (m, 1H), 3.50 (s, 3H), 3.40 (s, 3H), 3.23 (s, 3H), 3.30 (m, 2H), 3.12-3.04 (m, 6H), 2.84 (m, 4H), 2.70 (s, 3H), 2.43 (m, 1H), 2.20-0.70 (m, 86 H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 173.8, 173.7, 173.5, 173.4, 171.6, 171.2, 171.1, 170.3, 170.3, 170.1, 170.0, 136.7, 128.6, 128.4, 128.0,66.5, 64.3, 60.3, 58.8, 57.8, 57.8, 57.5, 55.4, 55.3, 55.2, 48.7, 48.5, 48.1, 45.0, 41.1, 40.5, 39.4, 38.9, 37.4, 35.9, 35.8, 33.9, 32.6, 31.5, 31.3, 31.1, 29.7, 29.7, 29.5, 29.4, 29.2, 29.2, 29.1, 29.0, 26.7, 25.3, 24.8, 24.8, 24.6, 24.4, 23.8, 23.7, 23.6, 23.4, 23.8, 23.7, 23.6, 23.4, 21.8, 21.7, 21.1, 21.0, 20.3, 19.8, 18.6, 18.3, 18.1, 16.8, 15.8, 14.1, 9.8; MALDI-MS calcd for $C_{78}H_{134}N_{12}O_{14}$ [M+Na]: 1486.0142, found 1486.0031.



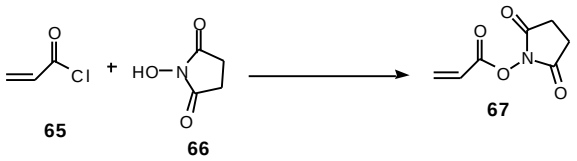
62 63

Cyclosporin A-linker 63: A mixed solution of starting Cbz-Cyclosporin A-linker **62** (206.6 mg, 0.14 mmol), Pd/C (10%, 68 mg) in 12 mL ethanol was degassed by connecting with water aspirator then flashed the flask with hydrogen balloon. Do this process three times to make sure the flask is filled with Hydrogen instead of air. After the reaction was continuing under Hydrogen for 1.5 h, the reaction mixture was filtered through celite and concentrated in vacuo. Flash chromatography with Et_3N deactivated SiO_2 eluting with EtOAc:MeOH (1:0→0:1) to give product **63** (117.4 mg, 62%) as a white solid: R_f = 0.16 (1:5-Et_2O:hexane); IR ($CHCl_3$) 3452, 2955, 2863, 2249, 1629, 1465 cm^{-1}; ^{1}HNMR(500 MHz, $CDCl_3$) δ 7.90 (d, *J* = 9.5 Hz, 1H), 7.61 (d, *J* = 7.5 Hz, 1H), 7.43 (d, *J* = 8.0 Hz, 1H), 7.12 (d, *J* = 8.0 Hz, 1H), 5.67 (dd, *J* = 10.5, 4.0 Hz, 1H), 5.43 (d, *J* = 5.5 Hz, 1H), 5.26 (dd, J = 11.5, 3.5 Hz, 1H), 5.10 (d, *J* = 11.0 Hz, 1H), 5.03-4.96 (m, 2H), 4.90 (m, 1H), 4.79 (m, 1H), 4.69 (d, J = 14.0 Hz, 1H), 4.60 (t, *J* = 8.5 Hz, 1H), 4.83 (m, 1H), 3.67 (t, *J* = 6.0 Hz, 1H), 3.47 (s, 3H), 3.35 (s, 3H), 3.22 (s, 3H), 3.17 (d, J = 14.0 Hz, 1H), 3.08-3.02 (m, 10 H), 2.90 (m, 2H), 2.66 (m, 6H), 2.40 (m, 1H), 2.13-0.76 9m, 82H), 0.67 (d, J = 6.5 Hz, 3H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 173.8, 173.7, 173.5, 173.3, 171.5, 171.2, 171.0, 170.5, 170.2, 170.0, 75.3, 60.3, 58.9, 57.6,

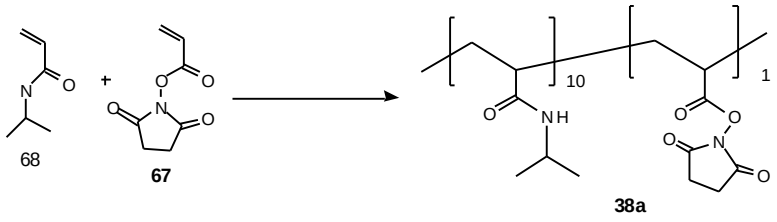
57.5, 55.3, 55.3, 55.2, 50.2, 48.6, 48.4, 48.1, 45.8, 45.0, 40.4, 40.0, 39.4, 38.9, 37.5, 36.4, 35.9, 34.0, 32.6, 31.5, 31.2, 31.0, 30.3, 29.7, 29.7, 29.6, 29.6, 29.5, 29.5, 29.4, 29.4, 29.1, 29.0, 28.9, 26.5, 25.2, 24.8, 24.7, 24.5, 24.3, 23.8, 23.7, 23.6, 23.4, 29.1, 29.0, 28.9, 28.1, 27.2, 26.5, 25.2, 24.8, 24.7, 24.5, 24.3, 23.8, 23.7, 23.6, 23.4, 21.8, 21.7, 21.1, 20.3, 19.8, 18.6, 18.2, 18.1, 17.0, 15.9, 14.1, 9.8, 8.8; HRMS (ESI) Calcd for $C_{70}H_{130}N_{12}O_{12}$ [M+H]: 1331.9931. Found: 1331.9096



Cyclosporin A-linker-polymer 64a: To an oven-dried 25 mL round bottom flask dissolve polymer (135 mg,, 0.11 mmol), DMAP (cat.) in 2 mL anhydrous THF. Then Cyclosporin A-linker **63** (13.8 mg, 0.010 mmol, 1.0 equiv.) in 1mL THF was added dropwise to the reaction flask. Finally 3 drop of Hunig's base was added to the flask. After the reaction was stirred for 12 h, *t*-butylamine (0.02 mL, 0.21 mmol,) was added to the reaction flask. After stirring at 24 °C for 9 h, the reaction mixture was filtered through celite column and the reaction liquid was concentrated in vacuo. The residue was purified by repetitive precipitation (THF, 3 mL and Hexane, 50 mL) four times to provide **64a** (27 mg, 70%). ^{1}HNMR (300 MHz, $CDCl_3$) δ 7.66 (d, 1H), 4.00 (s, 20H)



Ester 67: To a stirred solution of *N*-hydroxysuccinimide **66** (5.76 g, 50 mmol) and triethylamine (7.67 mL, 55 mmol) in 75 mL $CHCl_3$ at 0 °C, Acryloyl Chloride (5 g, 55 mmol) was added dropwisely during a 10 min period under nitrogen. After stirring for additional 20 min at 0 °C, the solution was washed with 120 mL ice-cold water and saturated sodium chloride solution, respectively. After the reaction mixture was concentrated to a volume of 15 mL, it was dried with anhydrous Mg_2SO_4, and filtered through celite, and 1.5 mL EtOAc and 10 mL of hexane were added slowly with stirring to the chloroform solution. Then the total solution was left at 4 °C for 2 h. The precipitated white solid was separated by filtration and washed with ice-cold 5 ml portion of a mixture of hexane: EtOAc (4:1), 5 mL portion of Hexane: EtOAc (9:1) and two additional 5 mL portions of Hexane, respectively. The product **67** (4.38 g, 52%) was dried over vacuum for 10 h. Spectroscopic data for this compound matched that previously reported.[51]



Polymer 38a: Into an oven dried 250 mL round bottom flask equipped with magnetic stirbar and reflux condenser was dissolved *N*-isopropylacrylamide **68** (1.67 g, 14.8 mmol) and Hydroxylsuccinimide ester **67** (250 mg, 1.48 mmol) in 40 mL *t*-butylalcohol. The reaction flask was evaculated and flushed with N_2 three times. Then the reaction mixture was heated to 75 °C in an oil bath. Into a separate vial was dissolved AIBN (5.8 mg) in 8 mL t-BuOH and evaculated and flushed with N_2 three times. After the reaction flask was stabilized under 75 °C for 1 h, the N_2 flushed AIBN solution was added to the reaction flask. After continue stirring at 75 °C for 23 h, the reaction solution was concentrated in *vacuo*. The residue was purified five times by repetitive precipitation with a THF/hexane solvent system (THF, 20 mL and hexane, 350 mL) to give product **38a** as a white solid. Spectroscopic data for this compound matched that previously reported.[51]

Analytical Affinity Chromatography with Sepharose-Cyclosporin A Amine Conjugates and Soluble Polymer- Cyclosporin A Amine Complex

Culturing the Jurkat-cells: The cell culture medium was defrosted by putting it in a 37 °C water bath for 40 min to let them warmed to around 37 °C. Into a 600 ml Falcon tissue culture flask was put 10 mL of stock Jurkat cell solution and 20 mL of flesh cell culture medium. Then the culture flask was put into the 37 °C CO_2 incubator. After three days, 5 mL of cell solution was sucked into another 600 mL Falcon tissue culture flask and an additional 75 mL flesh culture medium was added into the flask. After

another 72 h, the growing condition of the cell was checked under microscope and the amount of the cell was calculated. The cell culture was transferred into centrifuge tubes and centrifuged at 1,000 rpm for 20 min at 24 °C. After the supernatant was discarded, the PBS buffer was added to wash the cell. Then the solution was cetrifuged at 1,000 rpm for 10 min and the PBS supernatant was discarded.

The procedure of making the binding buffer (also used as lysis buffer): To a 2500 mL beaker was added the following reagents: 20 mM Tris-HCl power 3.152g; KCl 7.456g; 0.2% Triton X-100 2 mL; H_2O 800 mL. Then use 1N NaOH to adjust the pH of thesolution to 7.4. Then add more water to the buffer solution until the final volume of the solution to 1000 mL. Store in the refrigerator (4 °C). Everytime before use, add one protease inhibitor pill to 10 mL of buffer.

Affinity Experiment

Part One : Cell Lysis

The entire procedure was carried out on ice or in a cold room (4 °C)

1. All of the equipments were cooled in the cold room.
2. Cell pellet (3×10^8) was taken and 1.5 mL of binding buffer (20 mM Tris HCl, pH 7.4, 100 mM KCl, 0.2% Triton X-100, 2 μg/mL each of leupeptin, aprotinin, and soybean trypsin inhibitor) was added to the pellet.
3. The cell solution was homogenized 50 times within 15 minutes on ice.
4. The homogenate was centrifuged at 14, 000 rpm for 30 min. The supernatant was removed to a clean tube and the pellet was discarded.

5. the protein concentration was measured according to the manufacturer's direction. (Bio-Rad Protein assay Kit I)

Part Two : Affinity Binding

1. The lysate was aliquoted into six Eppendorf tubes and the amount of protein for each affinity tube was around 1.2 mg. Tube 1 was set as control, tube 2 as Sepharose-CsA binding, tube 3 as Sepharose-CsA/CsA competition, tube 4 as polymer-CsA binding, tube 5 as polymer-CsA/CsA competition, tube 6 as Polymer (Poly-N-isopropylacryamide poly-t-butylamine)
2. 40 µL of binding buffer was added to tube 1, 40 µL of Sepharose-CsA beads to tube 2, 40 µL of Sepharose-CsA beads to tube 3, 60 µL of polymer-CsA to tube 4, 60 µL of Polymer-CsA to tube 5, 60 µL of Polymer (Poly-N-isopropylacryamide poly-t-Butylamine) to tube 6. 200 µL of cell lysate was put into each tube. All tubes were put on the Belly Dancer at 4 °C rotating for 1.5 h.
3. 3 µL of CsA DMSO stock solution (17 mg/45 µL DMSO) was added to tube 3 and tube5 respectively. All the tubes were kept rotating for 0.5 h at 4 °C.
4. For tubes 1-3, they were spinned down at 8,000 rpm for 2 min at 4 °C. After the supernatant was removed and the beads was washed with 400 µL of binding buffer. Spinned down again and the supernatant was removed and the beads was washed with binding buffer again. The pellet beads was kept for future use. For tubes 4-6, they were warmed to 32 °C and then spinned down at 8,000 rpm for 3 min at 32 °C. The supernatant was removed and the residue was redissolved in 100 µL binding

buffer at 4 °C for 10 min. Then the solution was warmed to 32 °C and spinned down at 8,000 rpm for 3 min again. The supernatant was removed and the precipitate was kept.

5. 40 µL of SDS sample buffer was added to each tube. They were heated at 100 °C for 5 min and then the tubes were cooled to 25 °C. The cell tubes were spinned down for 5 minutes at 8,000 rpm at 32 °C. The solution was kept and the residue was discarded.
6. 18 µL solution from each tube was loaded onto the 12 % denaturing SDS-PAGE with wide protein molecule marker.

Part Three: Gel-staining with Coomassie Blue

The gel was stained with Coomassie Blue solution at room temperature.

1) Staining solution: Coomassie Blue R-250 2.5g; Methanol 454 mL; Acetic Acid glacial 92 mL, Water 454 mL 2) High methanol destaining solution: Methanol 454 mL; Acetic Acid glacial 75 mL; Water 471 mL 3) Low methanol destaining solution: Methanol 50 mL; Acetic Acid glacial 75 mL; Water 875 mL 4) Method for destaining: Gel in 100 mL staining solution for 40 minutes. Then shake the gel with 3 X100 mL High methanol destaining solution for 1.5 hours three times. Then the gel was put into 100 mL Low methanol destaining solution for 8 hours.

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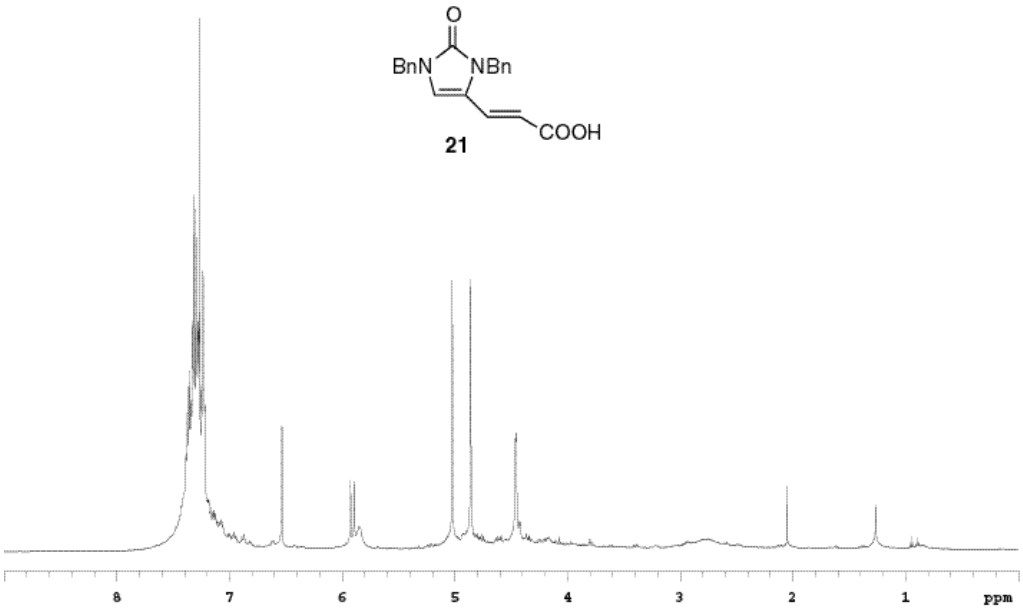
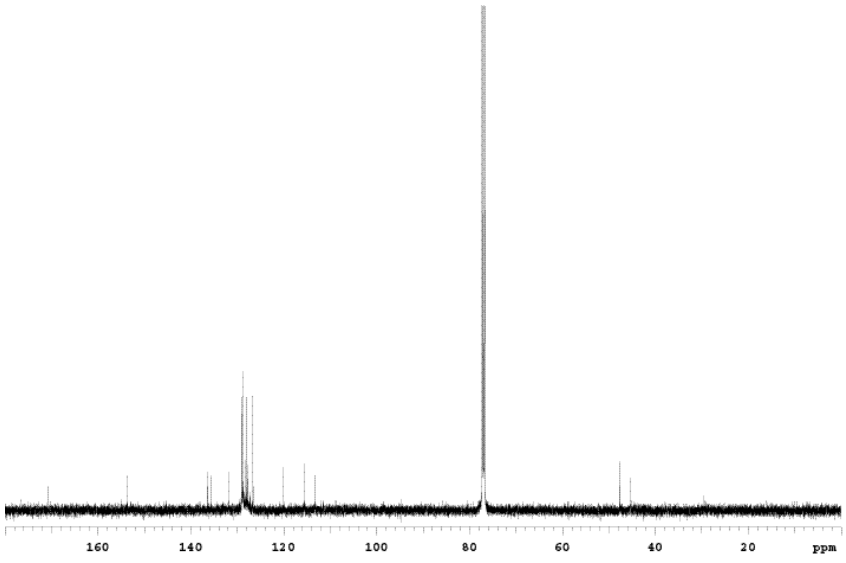
APPENDIX

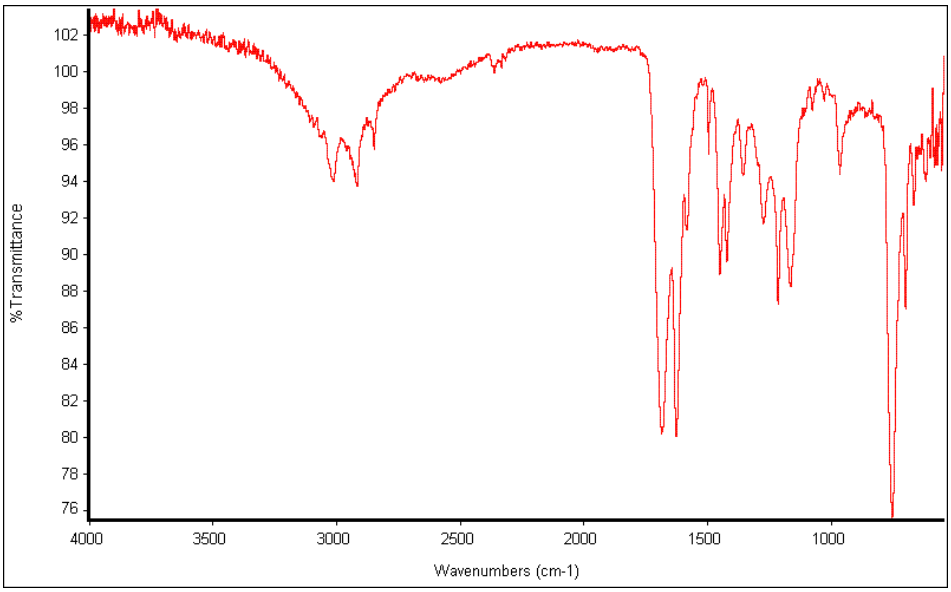
SELECTED SPECTRAL DATA



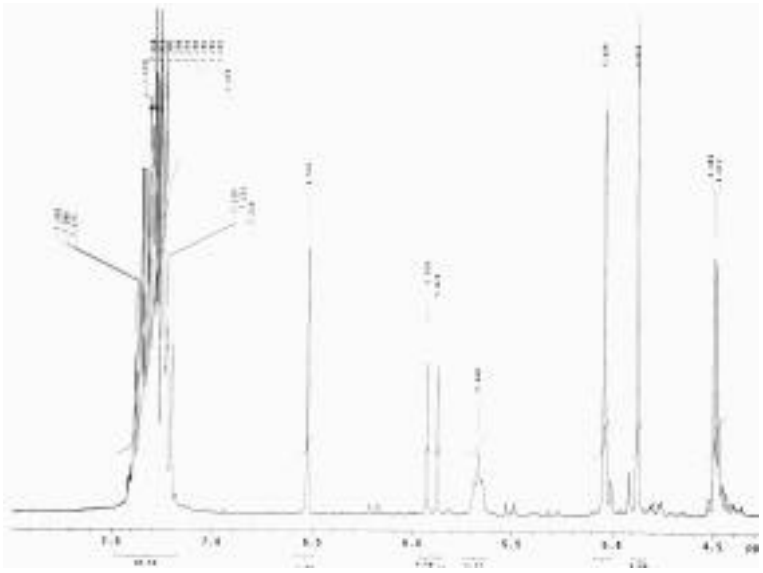
^{1}H NMR spectrum for α,β-unsaturated Acid **21** ($CDCl_3$)



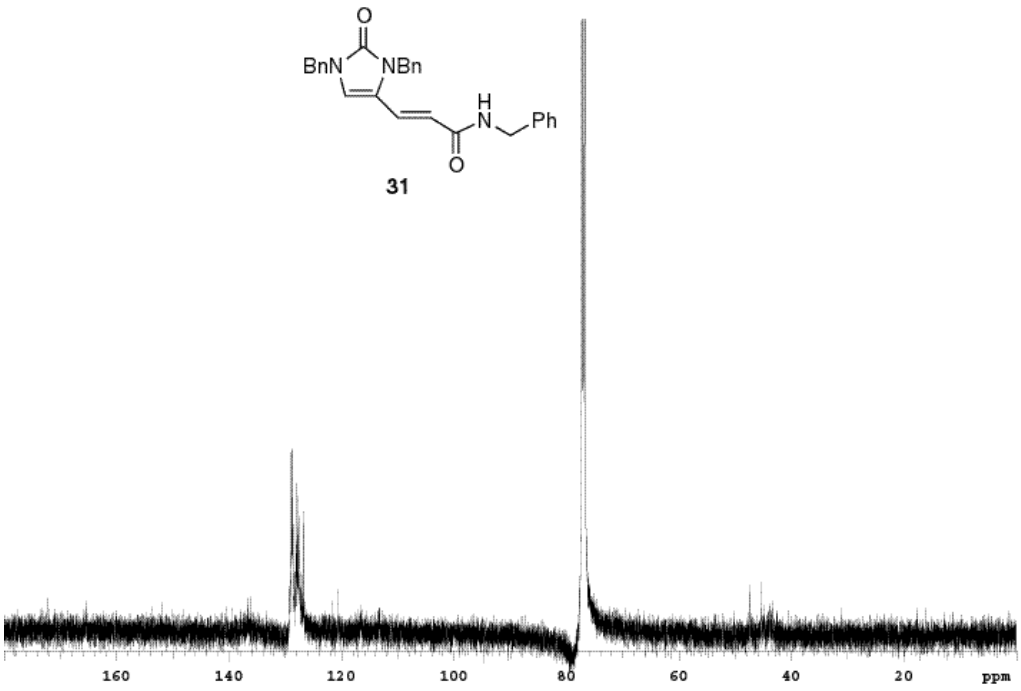
^{13}C NMR spectrum for α,β-unsaturated Acid **21** ($CDCl_3$)



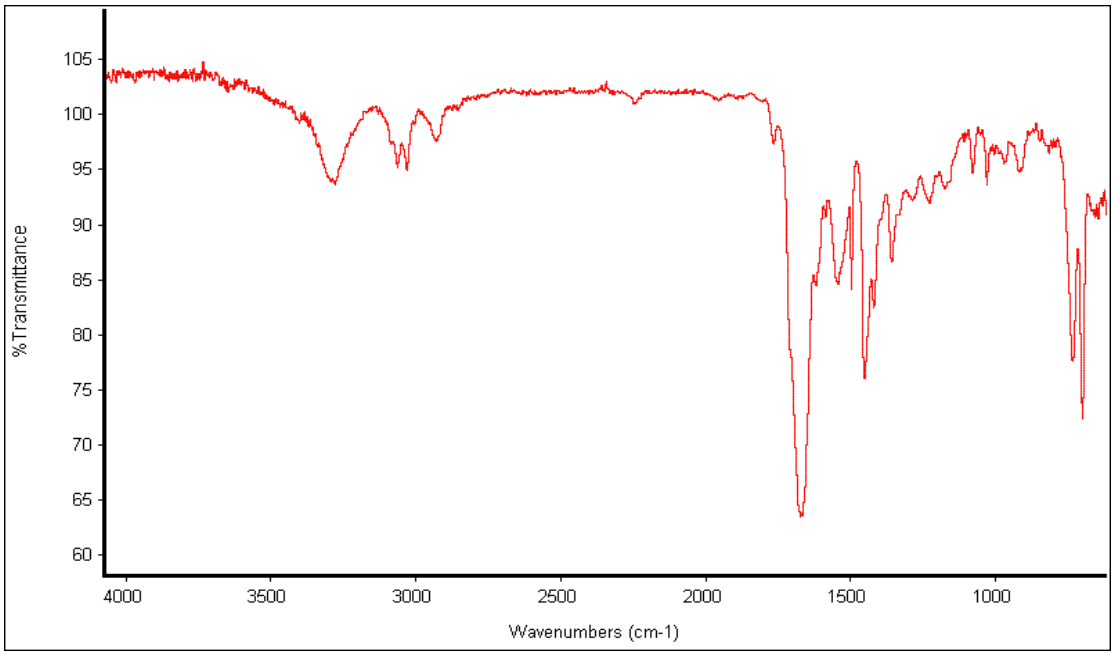
IR spectrum for α,β-unsaturated Acid **21**



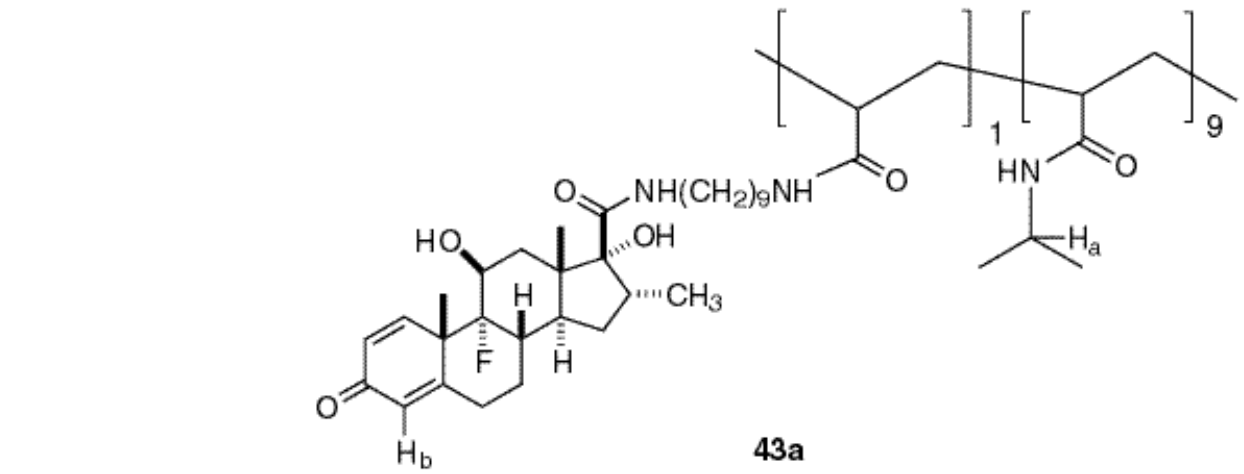
^{1}H NMR spectrum for α,β-unsaturated Amide **31** ($CDCl_3$)



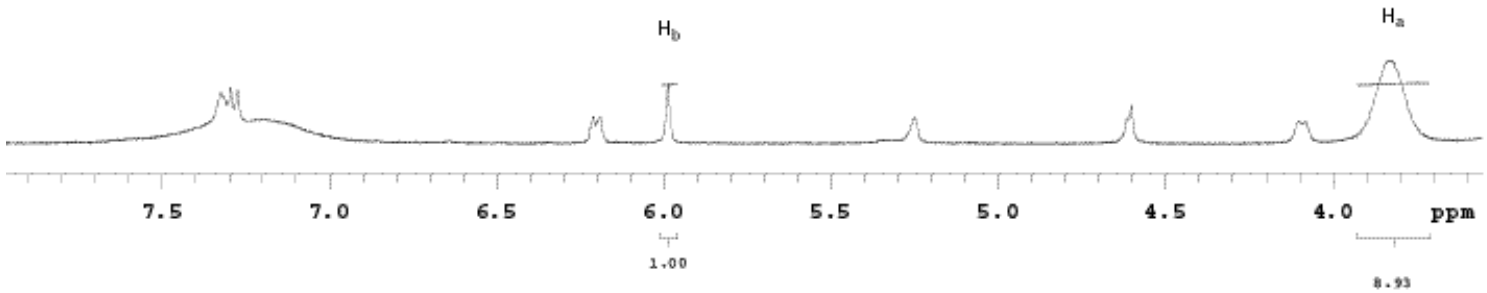
^{13}C NMR spectrum for α,β-unsaturated Amide **31** ($CDCl_3$)



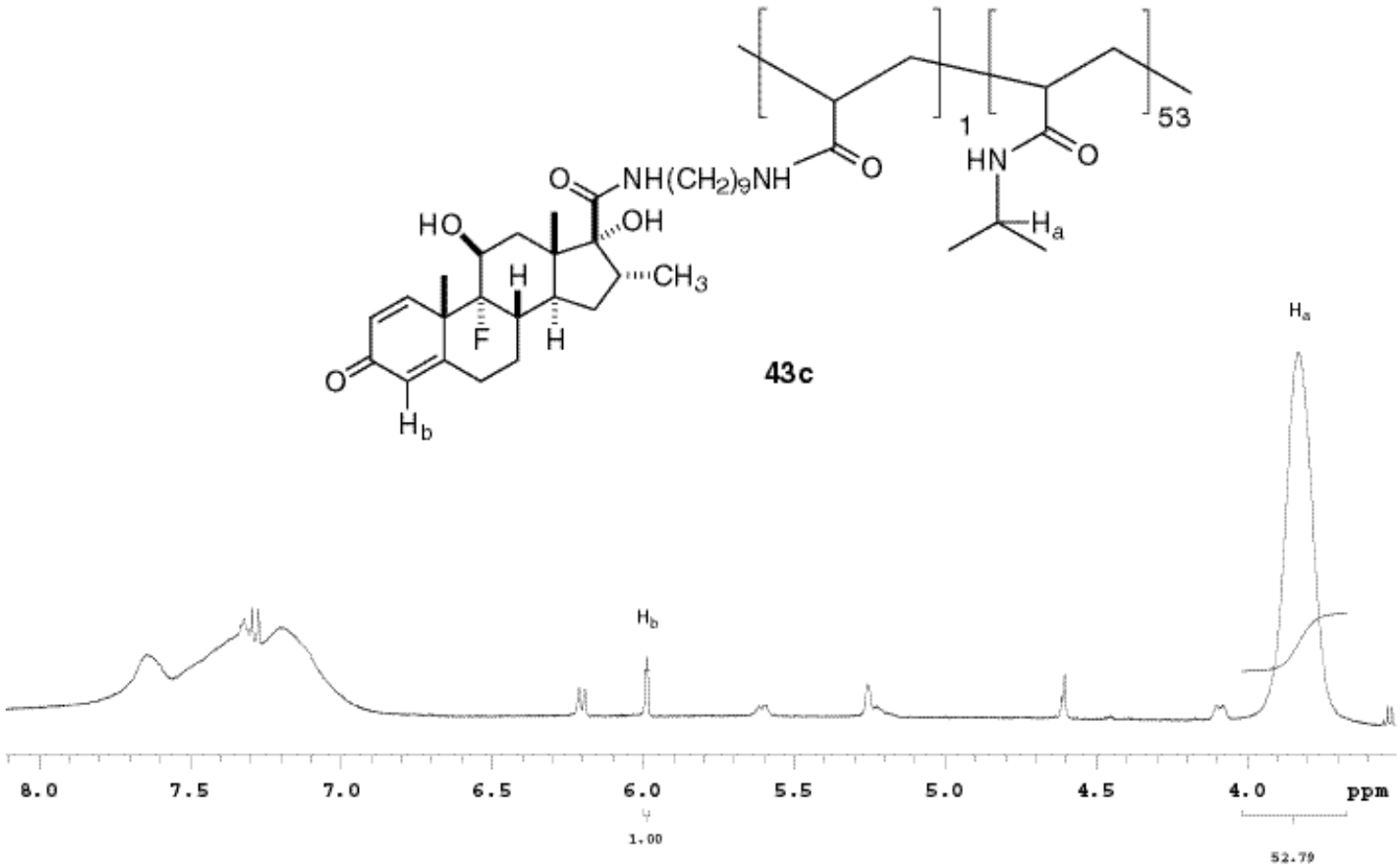
IR spectrum for α,β-unsaturated Amide **31** ($CDCl_3$)



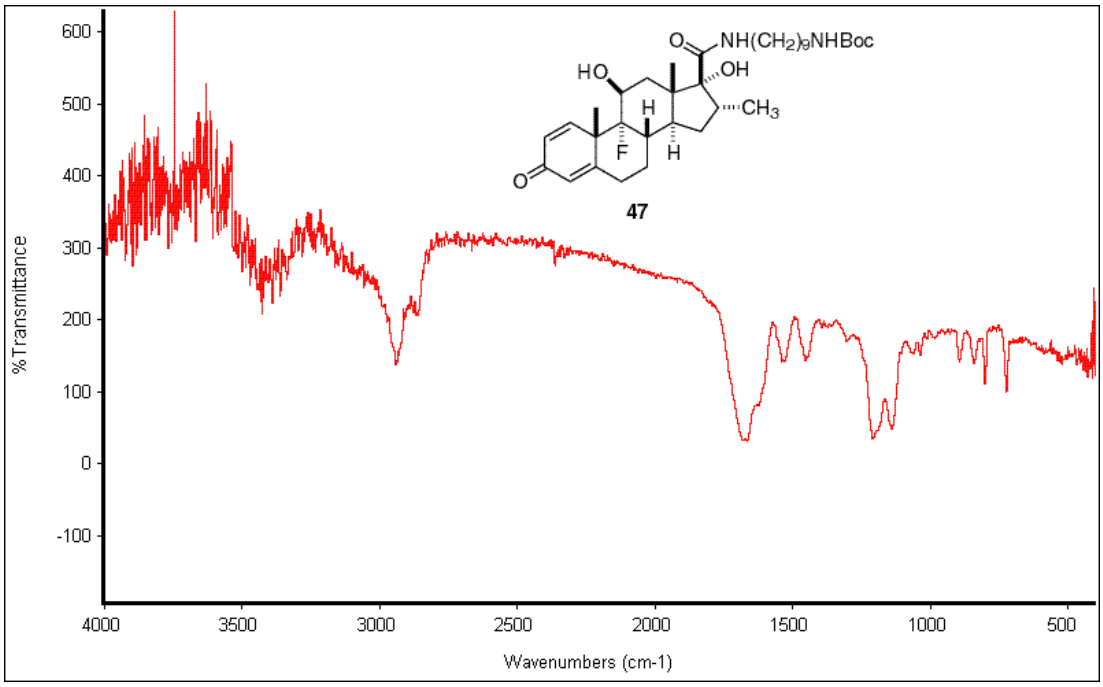
43a



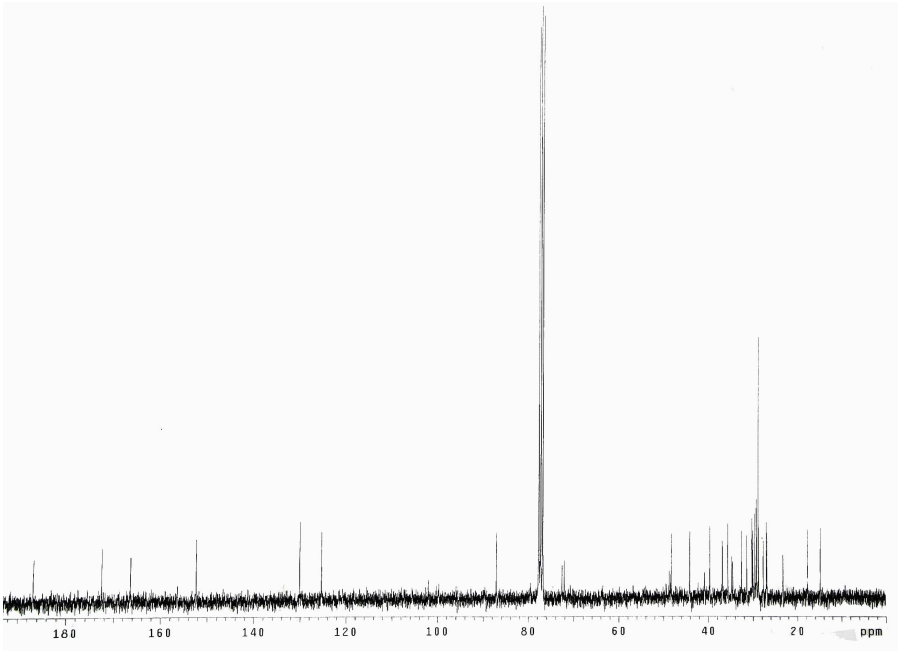
^{1}H NMR spectrum for Dexamethasone polymer macroligand **43a** (DMSO-d_6)



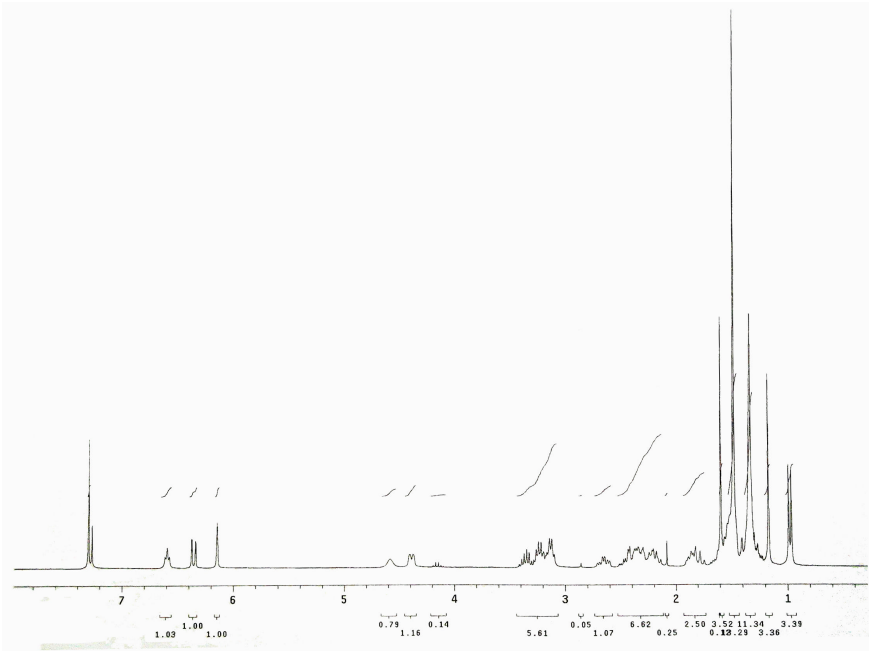
^{1}H NMR spectrum for Dexamethasone polymer macroligand **43c** (DMSO-d_6)



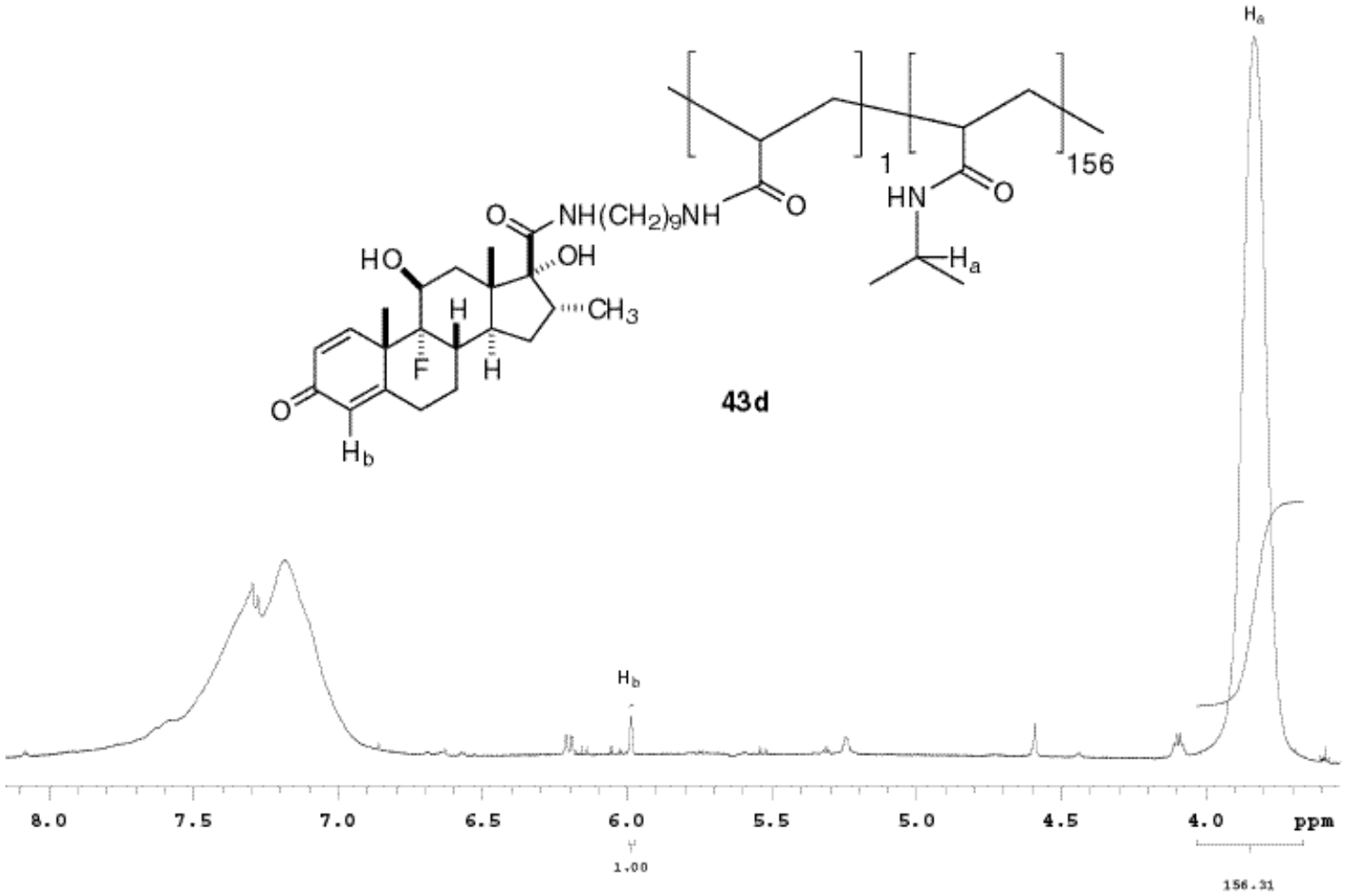
IR spectrum for Dexamethasone Amide **47** ($CDCl_3$)



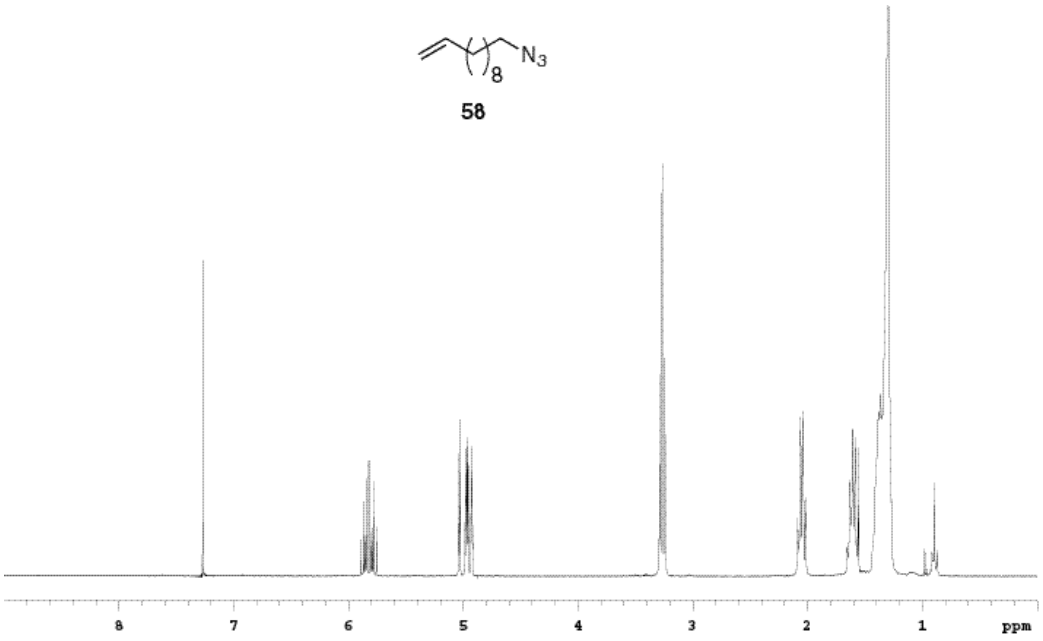
^{13}C NMR spectrum for Dexamethasone Amide **47** ($CDCl_3$)



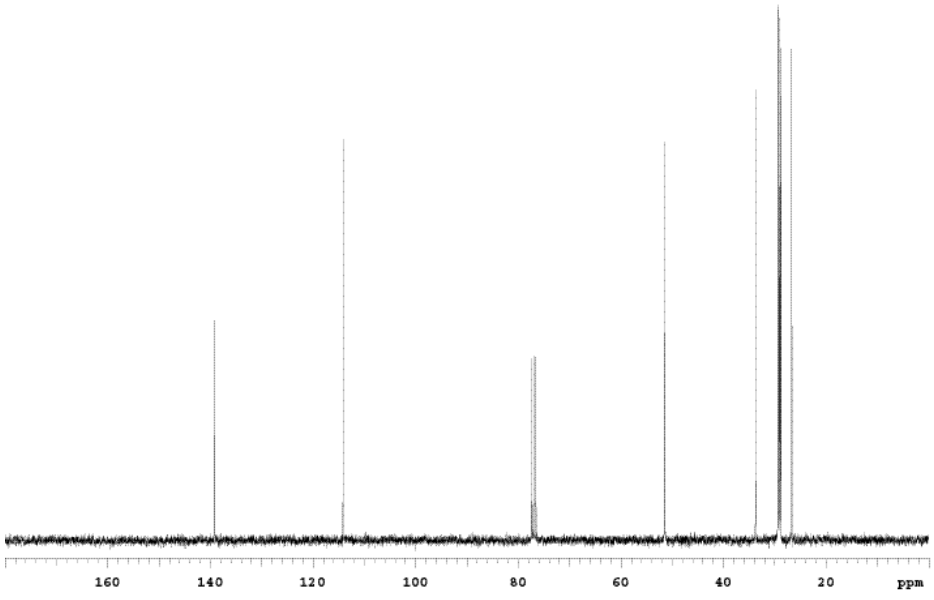
^{1}H NMR spectrum for Dexamethasone Amide **47** ($CDCl_3$)



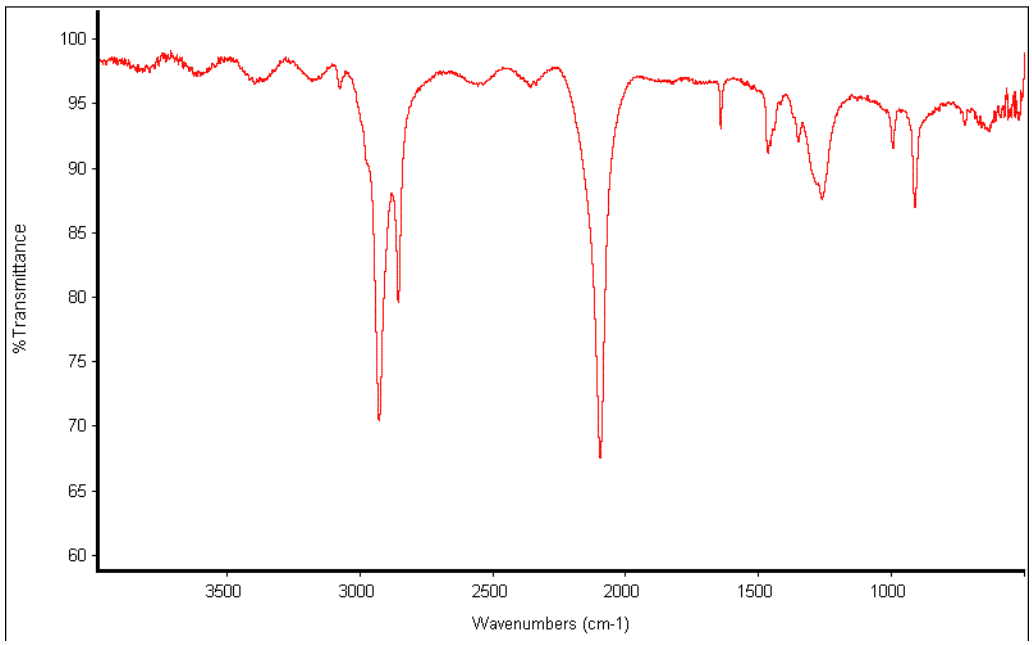
^{1}H NMR spectrum for Dexamethasone polymer macroligand **43d** (DMSO-d_6)



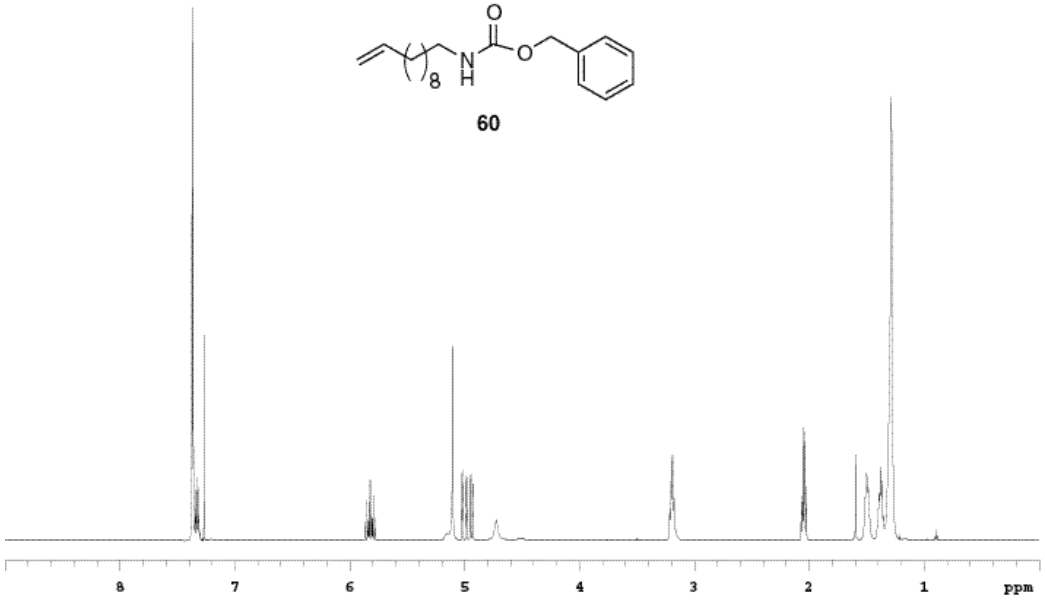
^{1}H NMR spectrum for ω-undecylenyl Azide **58** ($CDCl_3$)



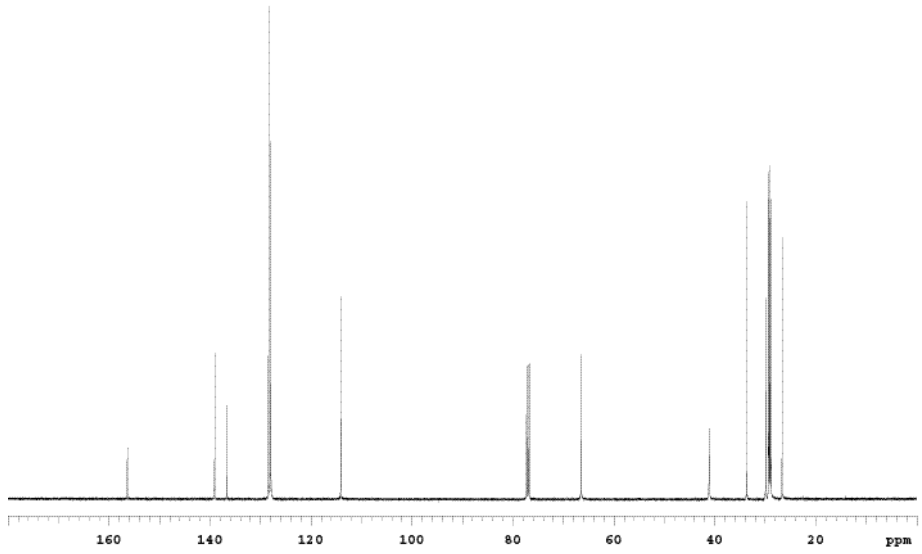
^{13}C NMR spectrum for ω-undecylenyl Azide **58** ($CDCl_3$)



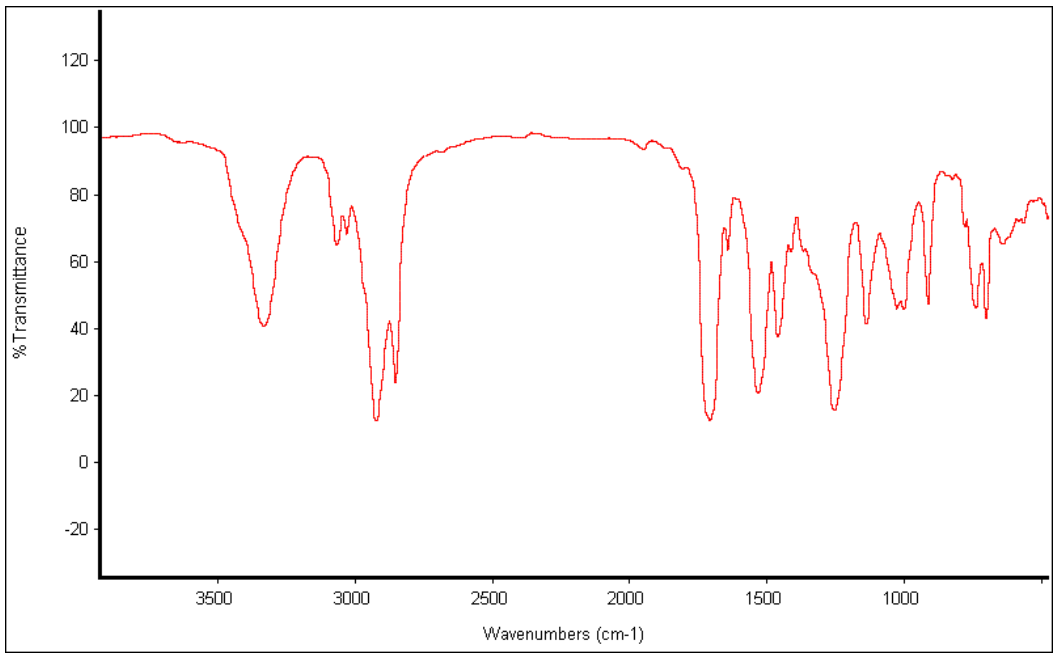
IR spectrum for ω-undecylenyl Azide **58**



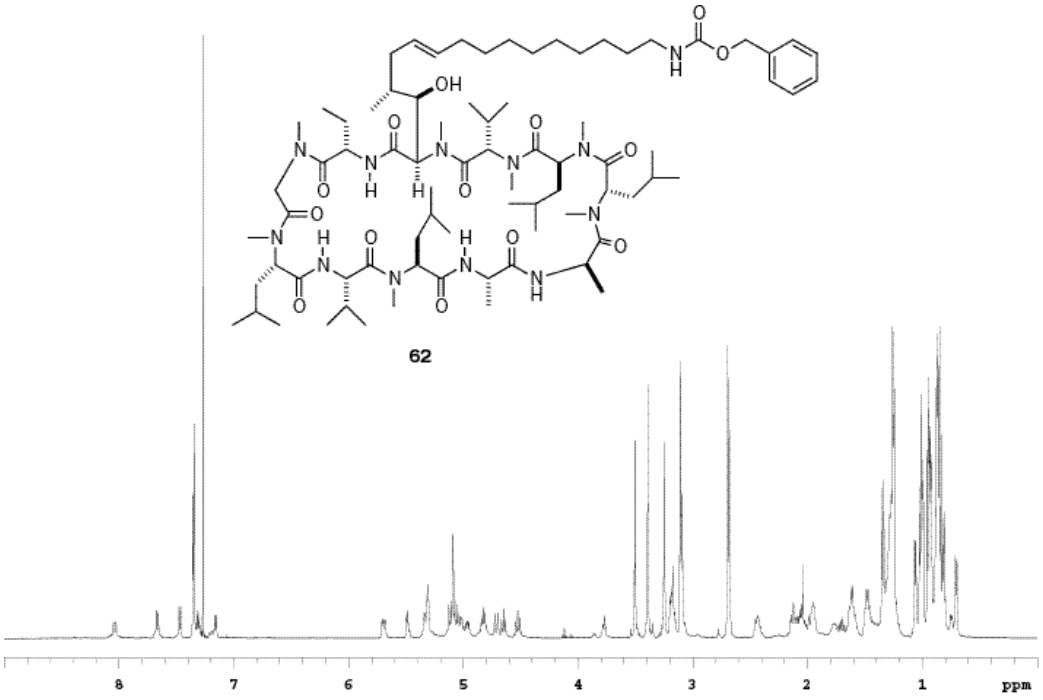
^{1}H NMR spectrum for *N*-Cbz-Aminal **60 (**$CDCl_3$)



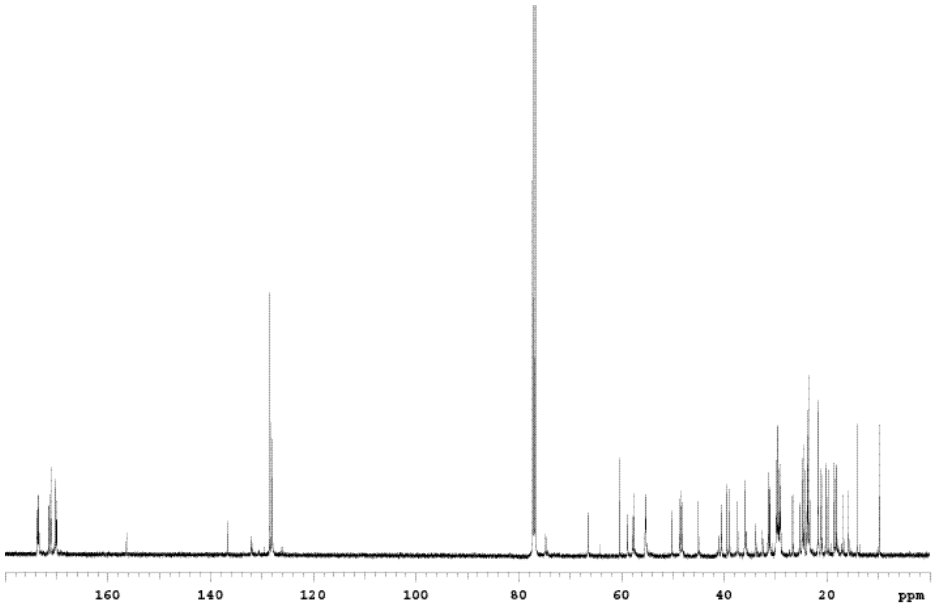
^{13}C NMR spectrum for *N*-Cbz-Aminal **60** ($CDCl_3$)



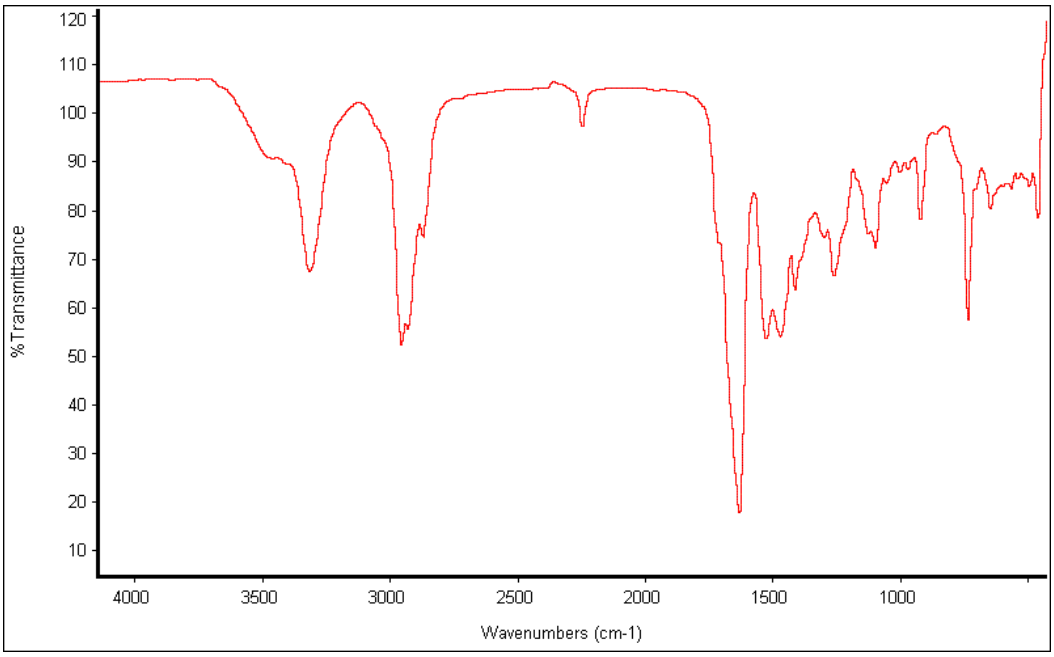
IR spectrum for *N*-Cbz-Aminal **60** ($CDCl_3$)



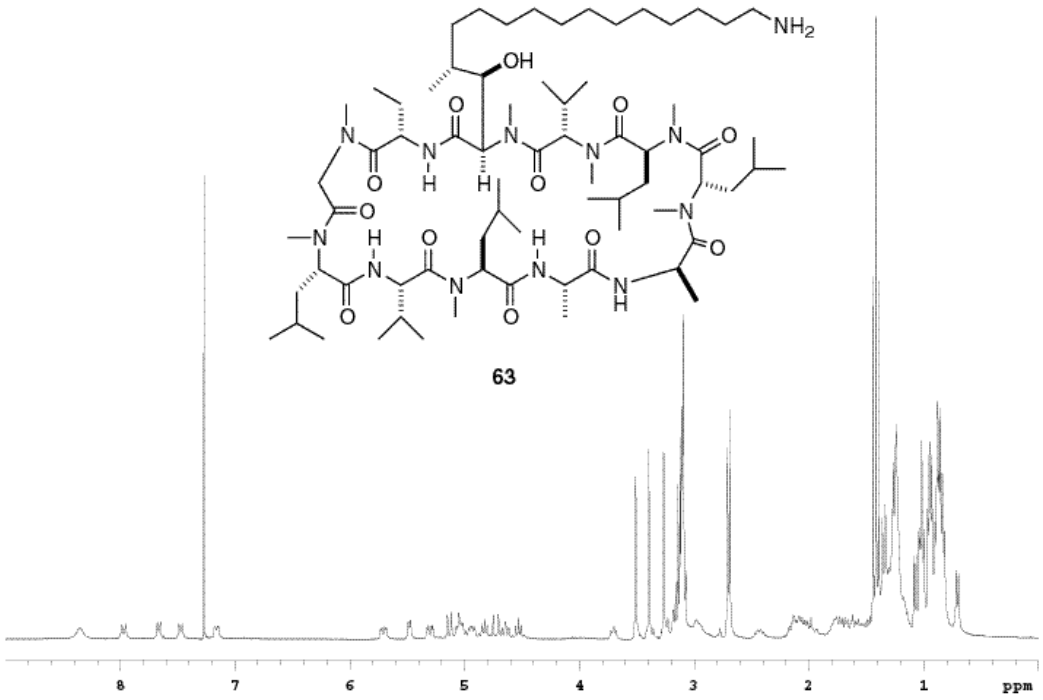
^{1}H NMR spectrum for Cbz Carbamate **62** ($CDCl_3$)



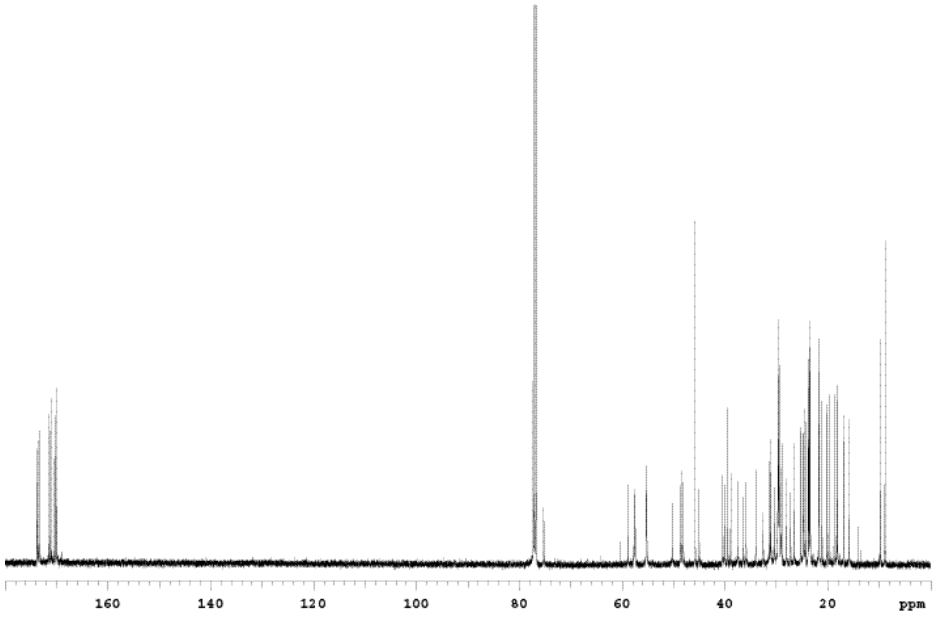
^{13}C NMR spectrum for Cbz Carbamate **62** ($CDCl_3$)



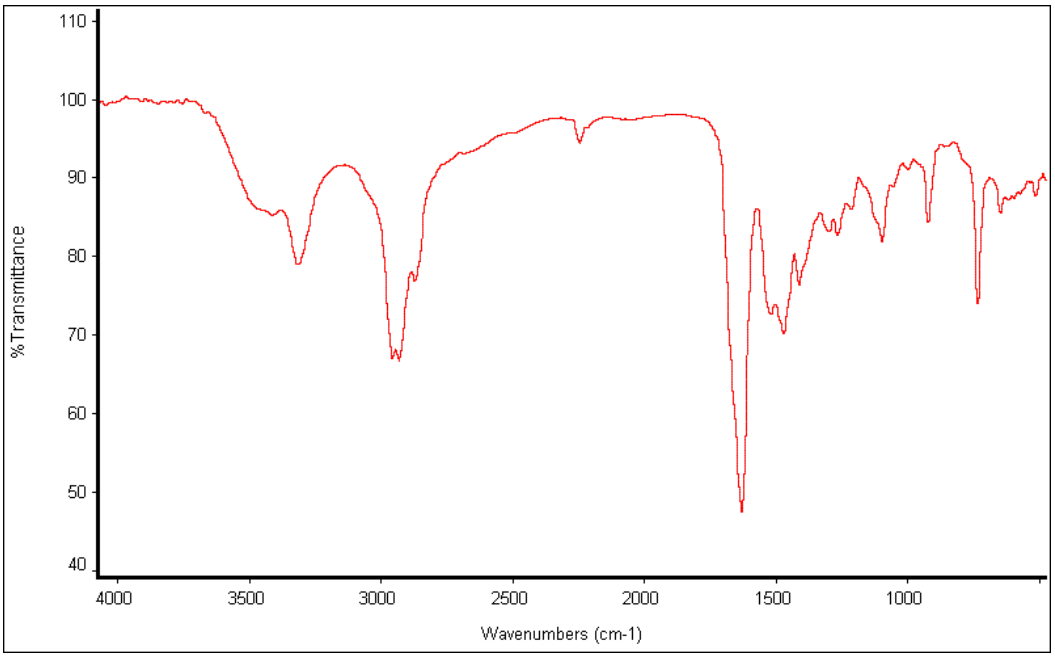
IR spectrum for Cbz Carbamate **62**



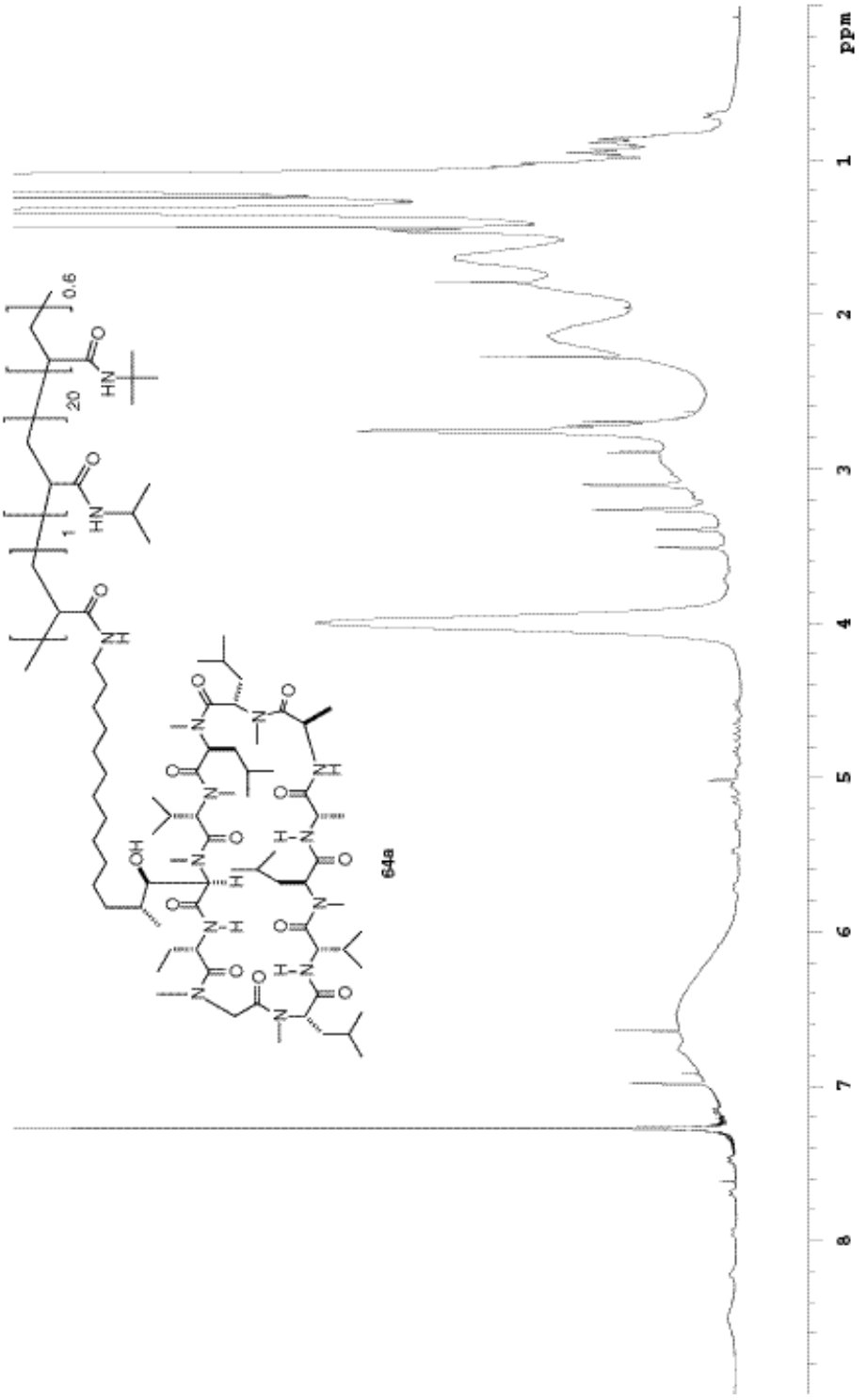
^{1}H spectrum for Cyclosporin A-linker **63** ($CDCl_3$)



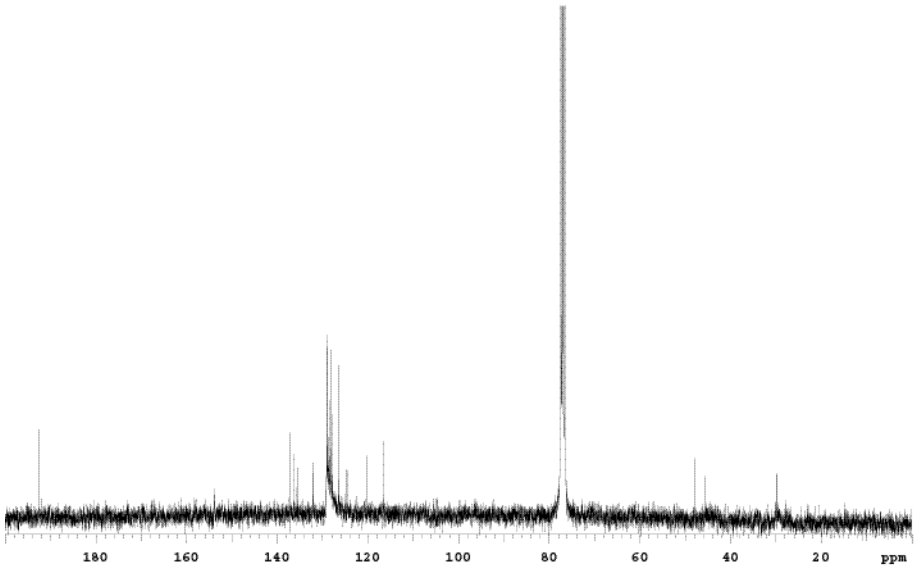
^{13}C spectrum for Cyclosporin A-linker **63** ($CDCl_3$)



IR spectrum for Cyclosporin A-linker **63** ($CDCl_3$)



^{1}H NMR spectrum for Cyclosporin A-linker-Polymer **64a** ($CDCl_3$)



^{13}C NMR NMR spectrum for α,β-unsaturated Aldehyde **69** ($CDCl_3$)

VITA

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